

AWARD NUMBER: W81XWH-05-1-0167

TITLE: Prostate Cancer Detection by Molecular Urinalysis

PRINCIPAL INVESTIGATOR: Christian Pavlovich, M.D
Brian Matlaga, M.D.

CONTRACTING ORGANIZATION: Johns Hopkins University
Baltimore, Maryland 21287

REPORT DATE: April 2011

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
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1. REPORT DATE 1 April 2011		2. REPORT TYPE Final		3. DATES COVERED 1 Apr 2005 – 31 Mar 2011	
4. TITLE AND SUBTITLE Prostate Cancer Detection by Molecular Urinalysis				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-05-1-0167	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Christian Pavlovich, M.D; Brian Matlaga, M.D. E-Mail: cpavlov2@jhmi.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Johns Hopkins University Baltimore, Maryland 21287				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT Prostate cancer is the most commonly diagnosed cancer and the second leading cause of cancer-related death in the United States. Serum tests for prostate cancer consist essentially of PSA and related isoforms, and when combined with digital rectal examination (DRE) are the mainstays of prostate cancer detection and local staging over the last decade. Recently however, it has become clear that urinary tests for prostate cancer may have a role in prostate cancer detection and prognostication. In this project, we have evaluated the feasibility of detection of prostate cancer by molecular urinalysis, and attempted to develop and validate new biomarkers for prostate cancer and prostatic disease detected on post-DRE urinalyses. Prostatic manipulation from DRE results in the shedding of prostatic material and even whole prostatic cells into the prostatic ducts and urethra: We have demonstrated that there is a cytokine profile associated with the prostatic fluid from men with large volume prostate cancer, and also found some cytokines to be elevated in prostatic fluid associated with benign prostatic hyperplasia (BPH). We performed studies on both prostatic fluids obtained ex vivo, and on urine samples obtained after DRE in the clinic. We pursued several of the proteins that appeared most interesting to us re: prostate cancer and BPH in such samples, including endoglin, IL-18 binding protein-a (IL18BP _a), and monocyte chemotactic protein-1 (MCP-1) and published manuscripts relating to these markers and their relationship to prostate pathology. For example, we found endoglin to be elevated in the urine of men with prostate cancer compared to those without, and it was also elevated in serum and was associated with burden of disease. MCP-1 was upregulated in the prostatic fluid and post-DRE urine from men with large prostate glands and from men with lower urinary tract symptoms (LUTS) on multivariate analyses controlling for age and prostate size, suggesting it may be a biomarker of LUTS and of an inflammatory phenotype in the prostate, though it appears downregulated in prostate cancer. We also found intact cells of prostatic origin in urinary cytopspins and using novel multiplex staining demonstrated prostate cancer cells in urine sediments, which has potential diagnostic application.					
15. SUBJECT TERMS prostate cancer, detection, urine, molecular analysis, urinalysis					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT UU	18. NUMBER OF PAGES 64	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U			19b. TELEPHONE NUMBER (include area code)

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INTRODUCTION:

Serum prostate-specific antigen (PSA) and digital rectal examination (DRE) remain the standard of care for prostate cancer screening despite their limited ability to detect occult prostate cancer. However, even 15% of men with a low PSA and normal DRE have prostate cancer on biopsy. In addition, the rate of false negative prostate biopsies is estimated to be between 20-35%. Clearly, more specific and sensitive tests are needed to spare unnecessary biopsies and better identify and prognosticate affected men with prostate cancer. The scope of this research is to study, develop, and optimize urinary biomarkers for the detection and prognostication of prostate cancer that may help discriminate benign from malignant conditions of the prostate.

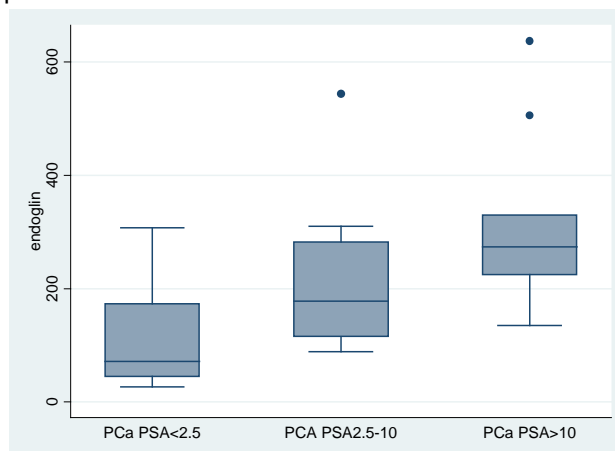
BODY:

We have collected patient urine specimens for biomarker analysis, and have collected and stored approximately 400 samples to date, of which some 200 remain available for future analyses. These are typically post-DRE collections from men with suspected or documented urologic disease, typically BPH or prostate cancer, and their urine is centrifuged and pellets separated from the supernatant. Urinary supernatants have been used for protein-level analyses, and the sediments/pellets have been used for DNA and cellular analyses. While our initial proposal was to evaluate the markers AMACR protein, GSTP1 (and other gene) methylation [Appendix, manuscript 2], and DD3/PCA3 in patient urine, over the years of the grant we have primarily worked on identifying and characterizing novel biomarkers and even intact prostate cells that we discovered in the post-DRE urine samples we collected. Through cytokine array and then confirmatory ELISA we have discovered a variety of markers associated with prostate cancer [Appendix manuscript 3], prostate cancer aggressiveness [Appendix manuscripts 5 and 7], and even markers associated with prostate gland size/urinary symptoms [Appendix manuscript 6]. We have also collaborated with researchers at other institutions and in other laboratories who have been interested in using our samples (Appendix, manuscript 1). Finally, we have identified prostate cancer cells in cytologic preparations of urinary sediment after DRE, with diagnostic relevance [Appendix manuscript 4].

Research:

We have continued to develop our own database of proteins that we found to be elevated in the prostatic fluid of prostate glands with high tumor volume at radical prostatectomy. We have queried this dataset in search of proteins that may be elevated in the prostate fluid of patients with *large volume prostate cancer*, and more recently in patients with *large prostate gland volumes* (but minimal cancer) at radical prostatectomy. The results of these analyses yielded molecules implicated with cancer and cancer progression (endoglin, and IL18BPα) [Appendix, manuscripts 5 and 7] and inflammation (monocyte chemotactic protein-1 (MCP-1; CCL2)) [Appendix, manuscript 6].

Our work on endoglin and IL18BPα has been published and can be found in the Appendix (manuscripts 5 and 7). A colleague of ours Dr. Robert Veltri is continuing to pursue endoglin as a marker of prostate cancer and a recent dataset of his confirmed some of our findings re: this molecule as being a hallmark of prostate cancer:



R. Veltri, unpublished data

Endoglin protein from urine samples of men with prostate cancer

We have correlated endoglin and IL18BP α levels with both cancer presence and aggressiveness, and recent work from other laboratories has confirmed these associations¹⁻³. On the other hand, MCP-1 overexpression in the prostate appeared associated with increasing prostate gland *weight* rather than cancer, where levels of this cytokine are often low. Others have shown a correlation between MCP levels in serum with prostate cancer metastasis,^{4,5} but levels in localized disease are low. In our multivariable analyses accounting for age, PSA, and lower urinary tract symptoms (LUTS), we noted a correlation between this molecule in urine/prostate fluid and lower urinary tract symptoms (LUTS). This correlation held up when it was extended to a larger cohort of men who had radical prostatectomy for what proved to be very low volume, low grade prostate cancer, in whom we had accurate prostate size, urinary symptom scores, age, and PSA information. A significant association was found on multivariate analysis between urinary MCP-1 level (normalized for total protein) and LUTS ($p < 0.04$), and in addition there was a trend ($p = 0.06$) toward an association of MCP-1 levels with severe LUTS. This hypothesis-generating work, which suggests that MCP-1-mediated inflammation may be a key player in symptomatic BPH, was presented at our national annual meeting in 2009 as a podium presentation from which a manuscript is currently in preparation. Another laboratory has recently associated MCP levels in urine with prostatic inflammation, along the lines of what we have found with LUTS, suggesting commonality between pathologic prostate inflammation and LUTS.⁶

In order to further explore the possibility that MCP-1 is a regulator of prostatic growth, we performed in vitro analyses using prostate epithelial (PrEC) and prostate stromal (PrSC) cell lines. Our experiments showed that MCP-1 is indeed produced by prostatic stromal cells, but that its receptor (CCR2) is expressed by both PrEC and PrSC. Exogenous MCP-1 or stromal-cell derived MCP-1 directly stimulated the proliferation of prostatic epithelial cells in culture in a dose-dependent manner. This effect was suppressed by either of two different inhibitors of MCP-1, an antibody and a small molecule inhibitor (RS102895). This work has since been published (Appendix manuscript 6).

In addition to work at the protein level, we also procured urine samples after DRE and preserved them for cytologic analysis. We optimized a method of fresh collection and preservation into cytopsins, and then developed a multiplex immunohistochemical staining platform for apparent prostate cancer cells using a combination of markers specific for AMACR, Nkx3.1, anti-nucleolin, and DAPI [Appendix manuscript 4]. The results of our studies suggest that molecular urine cytology is possible for prostate cancer detection, though much further study and newer markers could likely improve on our preliminary findings. Recent work in the UK supports this concept, as that group has used post-DRE urinary sediments to identify a novel marker for cancer (minichromosome maintenance protein 5) in cells for cancer detection.⁷

Training: Both Dr. Pavlovich and Dr. Matlaga have been active in mentored study with Drs. Isaacs and Trock. Bi-monthly lab meetings are standard. Both trainees have completed the Course of Research Ethics. Dr. Pavlovich successfully completed biostatistics courses at the Bloomberg School of Public Health, and a set of courses entitled the "Science of Clinical investigation". Dr. Matlaga holds an M.P.H. degree.

KEY RESEARCH ACCOMPLISHMENTS:

- Characterization of the cytokine profile of cancerous prostatic fluids and correlation with cancer and inflammation status
- Identification of specific cytokine markers of prostatic inflammation, which can differentiate the types of inflammatory cells in the prostate
- Assessment of urinary and serum endoglin as a biomarker of prostate cancer
- Assessment of urinary and serum IL18BP α as a biomarker for prostate cancer
- Identification of MCP-1 as a biomarker (and possible therapeutic target) for BPH
- Association of MCP-1 with lower urinary tract symptoms (LUTS) in men with BPH
- Assessment of the molecular interactions of IL18BP α and MCP-1 on prostate and prostate cancer cells in vitro
- Development of modern molecular cytologic techniques for the detection of prostate cancer using clinical urine specimens

- Development of a bank of urine samples enriched in prostatic fluid (collected after DRE) in patients with and without prostate cancer and with a variety of benign prostatic conditions

REPORTABLE OUTCOMES:

List of Manuscripts from funded research (all in Appendix 1):

- 1) Markushin Y, Gaikwad N, Zhang H, Kapke P, Rogan EG, Cavalieri EL, Trock BJ, **Pavlovich C**, Jankowiak R: Potential biomarker for early risk assessment of prostate cancer. *Prostate* 66(14): 1565-71, 2006.
- 2) Rogers CG, Gonzalgo ML, Yan G, Bastian PJ, Chan DY, Nelson WG, **Pavlovich CP**: High concordance of gene methylation in post-digital rectal examination and post-biopsy urine samples for prostate cancer detection. *J Urol* 176 (5): 2280-4, 2006.
- 3) Fujita K, Ewing CM, Sokoll LJ, Elliott DJ, Cunningham M, De Marzo AM, Isaacs WB, **Pavlovich CP**: Cytokine Profiling of Prostatic Fluid From Cancerous Prostate Glands Identifies Cytokines Associated with Extent of Tumor and Inflammation. *Prostate* 68(8):872-82, 2008.
- 4) Fujita K, **Pavlovich CP**, Netto GJ, Konishi Y, Isaacs WB, Ali S, De Marzo A, Meeker AK. Specific detection of prostate cancer cells in urine by multiplex immunofluorescence cytology. *Hum Pathol* 40(7):924-33, 2009.
- 5) Fujita K, Ewing CM, Chan DYS, Mangold LA, Partin AW, Isaacs WB, **Pavlovich CP**. Endoglin (CD105) as a Urinary and Serum Marker of Prostate Cancer. *Int J Cancer* 124(3):664-9, 2009.
- 6) Fujita K, Ewing CM, Getzenberg RH, Parsons JK, Isaacs WB, **Pavlovich CP**. Monocyte chemotactic protein-1 (MCP-1/CCL2) is associated with prostatic growth dysregulation and benign prostatic hyperplasia. *Prostate* 70(5):473-81, 2010.
- 7) Fujita K, Ewing CM, Isaacs WB, **Pavlovich CP**. Immunomodulatory IL-18 binding protein is produced by prostate cancer cells and its levels in urine and serum correlate with tumor status. *Int J Cancer* 29(2): 424-32, 2011.

List of Meeting Abstracts from funded research:

- 1) Mid-Atlantic American Urological Association, 2005: High Concordance Between Post-DRE and Post-Biopsy Urine Samples for Gene Methylation using MSP Analysis. Craig G Rogers, Gai Yan, Mark L Gonzalgo, David Chan, William G Nelson, **Christian P Pavlovich**
- 2) Mid-Atlantic American Urological Association, 2008: IL18 Binding Protein Is Produced By Prostate Cancer Cells And Its Levels In Urine And Serum Correlate With Tumor Status. Kazutoshi Fujita, Charles M Ewing, Alan W Partin, William B Isaacs, **Christian P Pavlovich**
- 3) Genitourinary Cancers Symposium, ASCO 2008 Abstract 54: Endoglin (CD105) as a potential urinary marker for prostate cancer detection. K. Fujita, **C. Pavlovich**.
- 4) Genitourinary Cancers Symposium, ASCO 2008 Abstract 273: Molecular Urine Cytology for Prostate Cancer Detection. K. Fujita, **C. Pavlovich**.
- 5) American Urological Association, 2009. Prostatic Monocyte Chemotactic Protein-1 (MCP-1): A Novel Biomarker for Lower Urinary Tract Symptoms Associated with Benign Prostatic Hyperplasia J. Kellogg Parsons, Kazutoshi Fujita, Charles M. Ewing, William B. Isaacs, **Christian P. Pavlovich**
- 6) American Urological Association, 2009. Monocyte chemotactic protein-1 (MCP-1:CCL2) is associated with prostatic growth dysregulation. Kazutoshi Fujita, Charles M Ewing, Robert H Getzenberg, J. Kellogg Parsons, William B Isaacs, **Christian P Pavlovich**.
- 7) American Urological Association, 2009. IL18 binding protein is produced by prostate cancer cells and its levels in urine and serum correlate with tumor status. Kazutoshi Fujita, Charles M. Ewing, William B. Isaacs, **Christian P. Pavlovich**.

Repository:

A urine repository of approximately 400 post-DRE urine sediments, still containing some 200, has been created. This is in use and so the actual number of specimens changes as new ones are added and old ones are used up by our or collaborative groups.

CONCLUSIONS:

Detection of prostate cancer by molecular urinalysis is feasible. We have described the cytokine profile of prostatic fluid associated with large volume prostate cancers, and studied the cytokine profile of large prostate glands with minimal cancer. We have found a variety of proteins, many inflammatory, that are elevated in these fluid specimens from both large volume prostate cancer cases (e.g. endoglin and IL18BP α), and also from cases with (little cancer) large prostate volume and lower urinary tract symptoms (MCP-1). We have explored the biology of some of these markers (e.g. IL18BP α and MCP-1). We have in addition developed a modern multiplex molecular cytologic assay for the detection of prostate cancer cells in voided urine specimens of men undergoing screening DRE. We also have created a post-DRE urine sample bank for subsequent analysis of these and other biomarkers as they become available that is available for collaborative use. Ultimately, our goal remains to discover, develop, and compare and contrast various modalities of molecular urinalysis for prostate cancer and prostate disease in the hopes of adding to the clinical utility and shortcomings of serum-based PSA analyses.

PERSONNEL:

- 1) **Kazutoshi Fujita, MD PhD, postdoctoral fellow**
- 2) **Hang Wang, PhD, postdoctoral fellow**

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- 1) Endoglin (CD105) and vascular endothelial growth factor as prognostic markers in prostatic adenocarcinoma. El-Gohary YM, Silverman JF, Olson PR, Liu YL, Cohen JK, Miller R, Saad RS. *Am J Clin Pathol.* 2007 Apr;127(4):572-9.
- 2) Preoperative plasma endoglin levels predict biochemical progression after radical prostatectomy. Svatek RS, Karam JA, Roehrborn CG, Karakiewicz PI, Slawin KM, Shariat SF. *Clin Cancer Res.* 2008 Jun 1;14(11):3362-6.
- 3) Endoglin regulates cancer-stromal cell interactions in prostate tumors. Romero D, O'Neill C, Terzic A, Contois L, Young K, Conley BA, Bergan RC, Brooks PC, Vary CP. *Cancer Res.* 2011 May 15;71(10):3482-93. Epub 2011 Mar 28
- 4) Activation of MCP-1/CCR2 axis promotes prostate cancer growth in bone. Lu Y, Chen Q, Corey E, Xie W, Fan J, Mizokami A, Zhang J. *Clin Exp Metastasis.* 2009;26(2):161-9. Epub 2008 Nov 11.
- 5) Targeting CCL2 with systemic delivery of neutralizing antibodies induces prostate cancer tumor regression in vivo. Loberg RD, Ying C, Craig M, Day LL, Sargent E, Neeley C, Wojno K, Snyder LA, Yan L, Pienta KJ. *Cancer Res.* 2007 Oct 1;67(19):9417-24.
- 6) Monocyte chemoattractant protein-1 and macrophage inflammatory protein-1 α as possible biomarkers for the chronic pelvic pain syndrome. Desireddi NV, Campbell PL, Stern JA, Sobkowiak R, Chuai S, Shahrara S, Thumbikat P, Pope RM, Landis JR, Koch AE, Schaeffer AJ. *J Urol.* 2008 May;179(5):1857-61; discussion 1861-2.
- 7) Diagnosis of prostate cancer by detection of minichromosome maintenance 5 protein in urine sediments. Dudderidge TJ, Kelly JD, Wollenschlaeger A, Okoturo O, Prevost T, Robson W, Leung HY, Williams GH, Stoeber K. *Br J Cancer.* 2010 Aug 24;103(5):701-7. Epub 2010 Jul 20.

APPENDIX:

FUNDING-RELATED PUBLICATIONS (7)

Potential Biomarker for Early Risk Assessment of Prostate Cancer

Y. Markushin,¹ N. Gaikwad,² H. Zhang,³ P. Kapke,⁴ E.G. Rogan,²
E.L. Cavalieri,² B.J. Trock,⁵ C. Pavlovich,⁵ and R. Jankowiak^{1*}

¹Department of Chemistry, Kansas State University, Manhattan, Kansas

²Eppley Institute for Research in Cancer, University of Nebraska Medical Center, Omaha, Nebraska

³Department of Chemistry, Iowa State University, Ames, Iowa

⁴Office of Biotechnology, Iowa State University, Ames, Iowa

⁵Brady Urological Institute, The Johns Hopkins School of Medicine, Baltimore, Maryland

BACKGROUND. Catechol estrogen quinones (CEQ) derived from 4-hydroxyestrone (4-OHE₁) and 4-hydroxyestradiol (4-OHE₂) react with DNA to form depurinating—N7Gua and—N3Ade adducts. This damage leads to mutations that can initiate breast and prostate cancer. To determine whether this damage occurs in humans, urine samples from men with prostate cancer and benign urological conditions, and healthy controls were analyzed. The objective was determining whether any of the cancer patients had formed the depurinating 4-OHE₁(E₂)-1-N3Ade adducts.

METHODS. The adducts were extracted from samples by using affinity columns equipped with a monoclonal antibody developed for detecting 4-OHE₁(E₂)-1-N3Ade adducts. Eluted extracts were separated by capillary electrophoresis with field-amplified sample stacking and/or ultraperformance liquid chromatography. Absorption/luminescence spectroscopies and mass spectrometry were used to identify the adducts.

RESULTS. 4-OHE₁-1-N3Ade was detected at higher levels in samples from subjects with prostate cancer (n = 7) and benign urological conditions (n = 4) compared to healthy males (n = 5).

CONCLUSION. This is the first demonstration that CEQ-derived DNA adducts are present in urine samples from subjects with prostate cancer. *Prostate* 66: 1565–1571, 2006.

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KEY WORDS: estrogens; DNA adducts; risk assessment; biomarkers; prostate cancer

INTRODUCTION

The natural estrogens, estrone (E₁), and estradiol (E₂), are metabolized at the 2- or 4-position with the formation of catechol estrogens, which, in turn, are metabolically oxidized into catechol estrogen quinones (CEQ). The latter have been implicated in the etiology of human breast cancer by various research studies [1–5]. The reaction of the CEQ, in particular CE-3,4-Q, with DNA forms the depurinating 4-OHE₁(E₂)-1-N3Ade and 4-OHE₁(E₂)-1-N7Gua adducts [1,5,6]. These two adducts constitute >99% of the total adducts formed. The CE-2,3-Q form only small amounts of the depurinating 2-OHE₁(E₂)-6-N3Ade adducts [7]. These

adducts generate apurinic sites that may lead to cancer-initiating mutations [8–11], which transform cells [12,13], thereby initiating cancer.

Grant sponsor: The National Cancer Institute; Grant number: 2P01 CA49210-12.

*Correspondence to: Prof. R. Jankowiak, Department of Chemistry, Kansas State University, Manhattan, KS 66502.

E-mail: ryszard@ksu.edu

Received 19 April 2006; Accepted 6 June 2006

DOI 10.1002/pros.20484

Published online 7 August 2006 in Wiley InterScience

(www.interscience.wiley.com).

Exposure to estrogens is a well-established risk factor for breast cancer [14]. The possible role of estrogens in prostate cancer is thus far less well established than in breast cancer. Limited evidence exists of an association between estrogens and risk of prostate cancer [15]. One important piece of evidence is the higher level of circulating estrogens observed in African-American men, who have a twofold higher risk of prostate cancer, compared to European-American men [16]. More direct evidence in support of the role of estrogens in prostate carcinogenesis comes from experiments using Noble rats treated with testosterone plus E_2 [17,18]. This combined treatment induces ductal adenocarcinoma of the prostate in 100% of the rats [17], whereas treatment with only testosterone causes prostate cancer in only 40% of the rats. Treatment with 5α -dihydrotestosterone, which unlike testosterone cannot be converted to E_2 , results in only a 4% incidence of prostate cancer. These carcinomas have been suggested to arise from estrogen-induced initiation and testosterone-produced promotion of the prostate tissue [15].

In our earlier work, CE-3,4-Q-derived DNA adducts were identified in tissue extracts from breast cancer patients [19]. In this case, samples were analyzed by CE interfaced with room-temperature absorption and low-temperature (laser-excited) phosphorescence spectroscopies. The level of the 4-OHE₁-1-N3Ade in the breast tissue extracted from a patient with breast carcinoma (8.40 ± 0.05 pmol/g of tissue) was larger by a factor of about 30 than the level in the breast tissue sample from a woman without breast cancer (0.25 ± 0.05 pmol/g of tissue) [19]. Although more breast tissue samples from women with and without breast cancer need to be studied, these results suggested that the -N3Ade adducts could serve as biomarkers to predict the risk of breast cancer.

To determine whether this type of DNA damage occurs in men, urine samples from subjects with prostate cancer, benign tumors, benign prostate hyperplasia, and a urological condition, as well as healthy males, were analyzed in a blind study. A primary objective was determining whether any of the subjects had formed 4-OHE₁(E₂)-1-N3Ade (Fig. 1), one of the major adducts formed by CE-3,4-Q. We showed that CE-3,4-Q-derived DNA adducts are present in human urine samples, and that their identification can be accomplished by a combination of techniques.

METHODS AND MATERIALS

Study Population and Samples

Single spot urine samples were obtained from two sources: (1) Healthy controls: samples were obtained

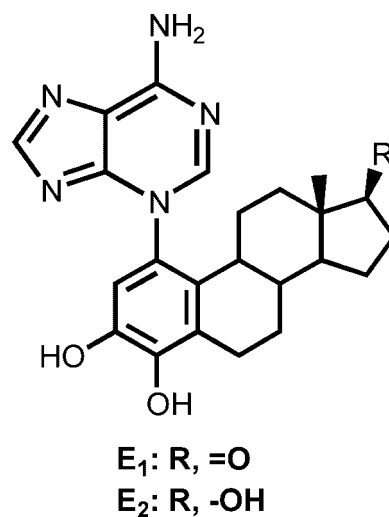


Fig. 1. The 4-OHE₁(E₂)-1-N3Ade adduct.

from men who had not been diagnosed with prostate cancer (Table I). (2) Urology clinic: samples were obtained from patients being seen at the urology clinic of Johns Hopkins Bayview Medical Center (Table I). Of these, two had benign urological conditions (erectile dysfunction or benign prostatic hyperplasia), two were undergoing prostate biopsy, and six were meeting with a surgeon to discuss surgery for newly diagnosed prostate cancer. Both of the patients undergoing prostate biopsy had urine collected prior to the biopsy; in both cases the biopsy was negative for prostate cancer. Urine samples were collected in a sterile container placed on dry ice and shipped untreated to the laboratory of Dr. Jankowiak by overnight mail.

Caution

CEQ are hazardous chemicals and should be handled carefully in accordance with NIH guidelines.

Chemicals and CEQ-DNA Adduct Standards

4-OHE₁ and 4-OHE₂ were synthesized according to Dwivedy et al. [20]. The 4-OHE₁- and 4-OHE₂-derived DNA adduct standards were synthesized as previously described [6,21]. Structural analysis of the above standards was accomplished via NMR and mass spectrometry (MS) [6,21]. Ultra-pure grade glycerol was obtained from Spectrum Chemical (Gardena, CA). The purity of standards for CEQ-derived DNA adduct standards, originally separated by HPLC, was verified in our laboratory by capillary electrophoresis (CE) and low-temperature luminescence spectroscopy. CEQ-derived DNA adducts, which are heat- and oxygen-sensitive, were kept for longer-term storage at -80°C under an inert atmosphere (N_2 or Ar). Samples were

TABLE I. Selected Characteristics of the Subject Population

Patient ID # in Figure 2	Diagnosis	Age	PSA	Smoking Status
3	Prostate Ca	68	4.2	Non-smoker
4	Prostate Ca	70	4.8	Smoker
5	Prostate Ca	47	5.6	Non-smoker
6	Prostate Ca	63	6.7	Non-smoker
8	Prostate Ca	61	3.6	NA
10	Prostate Ca	67	5.6	Non-smoker
11	Prostate Ca	53	NA	Non-smoker
1	Benign biopsy	67	7.3	NA
2	Erectile dysfunction	59	NA	NA
7	Benign biopsy	76	1.4	Non-smoker
9	BPH (post-TUNA) ^a	80	1.2	Smoker
12	Healthy control	21	^b	Non-smoker
13	Healthy control	23	^b	Non-smoker
14	Healthy control	17	^b	Non-smoker
15	Healthy control	21	^b	Non-smoker
16	Healthy control	35	^b	Non-smoker

^aNA, data not available; TUNA, trans-urethral needle ablation (a minimally invasive treatment for BPH).

^bNot measured.

dissolved in methanol:buffer (80:20), with the following buffer content: 0.1 M ammonium acetate and 1 mg/L ascorbic acid in nanopure water, pH 4.5. Tris[hydroxymethyl] aminomethane was purchased from Fisher Scientific (Fairlawn, NJ). Phosphoric acid and polyoxyethylene 8 cetyl ether (C₁₆E₈) were obtained from Sigma-Aldrich (St. Louis, MO).

Monoclonal Antibodies (MAb)

Ovalbumin (OA) and keyhole limpet hemocyanin (KLH) were purchased from Pierce Biotechnology, Inc., Rockford, IL. Delbecco's Modified Eagle medium and horse serum were purchased from Mediatech, Inc., Herndon, VA, and Valley Biomedical, Inc., Winchester, VA, respectively. *N*-(9-Fluorenyl) methoxycarbonyl multiple antigenic peptides (Fmoc MAP) resin was purchased from Applied Biosystems, Foster City, CA. Well-established methods [22] were used to generate an immune response in mice. The 4-OHE₁-1-N3Ade linker (synthesized similarly to the 4-OHE₁-2-NAcCys-16 α , β -MCC linker [23, unpublished results]) was conjugated to KLH and used in an immunization protocol with 25 μ g of antigen/mouse/injection using Freund's incomplete adjuvant. Serum titers were established using 4-OHE₁-1-N3Ade conjugated to OA. Mouse spleen cells were fused with an equal number of SP2/O cells (40 million of each) and plated in 16 \times 96-well microtiter plates. When hybridoma wells started to turn yellow, the plates were screened using an ELISA

assay. Five hundred nanograms of OA-4-OHE₁-1-N3Ade linker in binding buffer (100 mM NaHCO₃, pH 9.3) was used to coat each well of a Nunc Maxisorb plate. Hybridoma cell lines were screened using 4-OHE₁-1-N3Ade conjugated to OA. Since there is no immunological cross reactivity between KLH and OA, positive hybridoma cell lines secreting antibody against 4-OHE₁(E₂)-1-N3Ade could be rapidly identified using OA-4-OHE₁-1-N3Ade.

Affinity columns were developed and used to purify MAb against 4-OHE₁-1-N3Ade, by passing 3 mL of medium from the selected hybridoma over the column. Then the columns were washed with 50 mL of PBS and antibody eluted with 100 mM acetic acid, pH 2.5. MAbs were concentrated by centrifugal filters (Millipore Corporation, Bedford, MA) using an Amicon Ultra 100,000 molecular weight cut. This allowed separating solutes from low MW compounds. The eluted antibody was isotyped using a kit specific for mouse IgG antibody, confirming that the antibody was of mouse origin and not from the horse serum used in growing the cells. This purified MAb (assigned as 15G8) was immobilized on an agarose bead column (Aminolink kit, Pierce Inc.) and used to detect 4-OHE₁-1-N3Ade in PBS buffer that was spiked with various concentrations of -N3Ade adduct (data not shown). We have demonstrated that the 15G8 MAb binds with high affinity to 4-OHE₁-1-N3Ade adducts ($K_a = 10^8$ /M), highly discriminating against a large spectrum of closely related CEQ-derived metabolites [unpublished results]. It has

been used here for detecting 4-OHE₁(E₂)-1-N3Ade adducts in urine samples. The immunoaffinity columns were used to capture and preconcentrate the hapten of interest from the urine samples.

Capillary Electrophoresis

The analysis of urine extracts was done using a P/ACE MDQ CE system (Beckman/Coulter, Fullerton, CA) with a photodiode array (PDA) detector for simultaneous detection of electropherograms and UV absorption spectra of separated analytes. A bare fused-silica capillary (Polymicro Technologies, Phoenix, AZ) with 21 cm effective length and 31.2 cm total length (75 μ m I.D. and 360 μ m O.D.) was used. The running buffer was 0.5% C₁₆E₈ in 0.25 mM Tris-phosphate (pH 3.5). Before injection, the solvent in the sample was evaporated under vacuum; the sample residue was then diluted with the same volume of 75 μ M H₃PO₄ solution. The same extract was also separated with an ISCO (Lincoln, NE) model 3140 Electropherograph System, and re-analyzed by low-temperature luminescence spectroscopy. The CEQ-derived DNA adduct standards, and the extracts from the immunoaffinity columns were analyzed with field-amplified sample stacking (FASS) conditions. FASS [24,25] was used for analyte preconcentration. To achieve reproducible and accurate stacking results, a water plug was injected into the capillary before the sample (at 0.2 psi for 12 sec) followed by the electrokinetic injection of urine extract sample at +10 kV for 30 sec. The applied electric field for separation was 480 V/cm, and the running temperature was 25°C. The absorption detection was set at the PDA mode to obtain the electropherograms at different UV wavelengths and the absorption spectra of the separated analytes. After each run, the capillary was rinsed with 0.1 M NaOH for 2 min, and running buffer for 5 min. Electropherograms were obtained in the absorbance mode. CE-separated DNA adducts were identified based on the characteristic migration times and corresponding absorption spectra. Various detection wavelengths for the CE electropherograms were utilized (e.g., 214, 260, and 276 nm).

Luminescence and Absorption Spectroscopy

Luminescence spectra were obtained using an excitation wavelength of 257 nm with a Lexel 95-SHG-257 CW laser. Emission was dispersed by a Model 218 0.3-m monochromator (McPherson, Acton, MA), equipped with a 300 G/mm grating, providing a resolution of \sim 1 nm. Spectra were detected with an intensified CCD camera (Princeton Instruments, Trenton, NJ) using gated and non-gated modes of detection. A fast shutter, operated by a Uniblitz driver control (model SD-12 2B), was synchronized with the CCD

camera (ICCD-1024 MLDG-E1) and used for time-resolved phosphorescence measurements. Using this setup, time-resolved phosphorescence spectra could be measured in 0.5 sec intervals with a gate width of 0.5 sec. To ensure good glass formation in off-line spectroscopic measurements, glycerol (50% by volume) was added to the samples in buffer just prior to cooling to 77 K in a liquid nitrogen optical cryostat with suprasil optical windows. Samples (ca. 20 μ L) were contained in suprasil tubes (2-mm i.d.). All spectra were background corrected.

UPLC and Mass Spectrometry

The sample was diluted 1:10 in 50% methanol in water and analyzed twice by ultraperformance liquid chromatography (UPLC). The parent-daughter transition used in analysis was $m/z = 420.1 > m/z = 296.0$, which was analyzed with a Waters Acquity binary solvent manager and Sample Manager Micromass and a Quattro Micro mass spectrometer. The gradient was 80% (H₂O, 0.1% formic acid), 20% (acetonitrile, 0.1% formic acid) to 79% (H₂O, 0.1% formic acid), 21% (acetonitrile, 0.1% formic acid) in 4 min, to 45% (H₂O, 0.1% formic acid), 55% (acetonitrile, 0.1% formic acid) in 6 min. Standard curves were established and quantitation was carried out using QuanLynx v4.0.

Statistical Methods

Mean levels of 4-OHE₁(E₂)-1-N3Ade adduct were compared between patient groups using the Student's *t*-test and analysis of covariance, the latter to permit adjustment for age. Linear regression was used to evaluate the correlation between 4-OHE₁(E₂)-1-N3Ade adduct levels and prostate specific antigen (PSA). All analyses were performed using SAS version 9.1 software (SAS Institute, Cary, NC).

RESULTS AND DISCUSSION

Detection of 4-OHE₁(E₂)-1-N3Ade Adducts in Urine Samples

Urine samples (20 mL each) from 16 subjects were analyzed in blind studies using different detection methods, as described above. Selected characteristics of the subjects are presented in Table I. All specimens were initially separated using affinity column purification, that is, the adducts of interest were extracted from the urine samples using home-built columns equipped with the 15G8 MAb. Eluted extracts from immunoaffinity columns were analyzed by laser-excited low-temperature phosphorescence spectroscopy and UPLC interfaced with tandem mass spectrometry (LC/MS/MS).

In addition, urine samples, after lyophilization and methanol extraction, were pre-concentrated and analytes therein were separated by CE with FASS and detected by absorbance-based electropherograms. A spiking procedure with synthesized DNA adduct-standards and absorption/luminescence spectroscopies were used to identify the biomarkers of interest.

In this initial study, the 4-OHE₁(E₂)-1-N7Gua adducts was not analyzed for two main reasons. First, development of a MAb to the N7Gua adduct would have delayed the study by 6 months or more. Second, studies on the mutagenicity of 4-OHE₂ and E₂-3,4-Q reveal only A to G transition mutations [8–11]. These results imply that the instantaneous depurination of N3Ade adducts generates error-prone repair of de-adenylated sites [8].

In Figure 2, the bars in row #1 correspond to the integrated (normalized) area of the absorbance-based CE electropherogram peaks assigned to 4-OHE₁(E₂)-1-N3Ade. Only the samples from the urology patients contained 4-OHE₁(E₂)-1-N3Ade adduct. Among the urology patients, there were no significant differences in mean 4-OHE₁(E₂)-1-N3Ade adduct levels between the patients with prostate cancer (mean = 86.9 pmole/mg) or the patients with benign urologic conditions (mean = 108.5 pmole/mg), $P = 0.665$. When the comparison was adjusted for age, there was no significant difference, $P = 0.385$. Among the eight subjects with recent PSA results, there was no correlation between

4-OHE₁(E₂)-1-N3Ade adduct levels and PSA ($P = 0.583$). The adduct level (normalized to creatinine concentration) varied from sample to sample with concentration levels of about 15–240 pmole per mg of creatinine. The identity of the adducts in samples #1–11 in row #1 was confirmed by low-temperature (77 K) luminescence spectroscopy, as shown by the bars in row #2 of Figure 2, which correspond to the integrated (normalized) area of the low temperature phosphorescence spectra obtained for urine samples #1–11 eluted from the immunoaffinity columns. Examples of the phosphorescence spectra obtained for samples #1, 4, and 6 are shown in the right inset of Figure 2; the red spectrum overlapping with the phosphorescence spectrum of sample #6 is that of the standard adduct. In fact, all spectra measured for samples #1–11 revealed emission identical to the phosphorescence spectrum of the 4-OHE₁(E₂)-1-N3Ade adduct standard [19] (data not shown), thus proving again that the analyte eluted from the 15G8-MAB based column corresponds to 4-OHE₁(E₂)-1-N3Ade. The amount of this adduct in samples #1–11 using low temperature phosphorescence-based calibration curves was about 10–150 pmole per mg of creatinine, depending on the sample. With a detection limit of about 10^{-9} M [19], 4-OHE₁-1-N3Ade adducts were not detected in samples #12–16 from healthy control subjects, in agreement with the CE/FASS results. The observed emission intensity was near the background level.

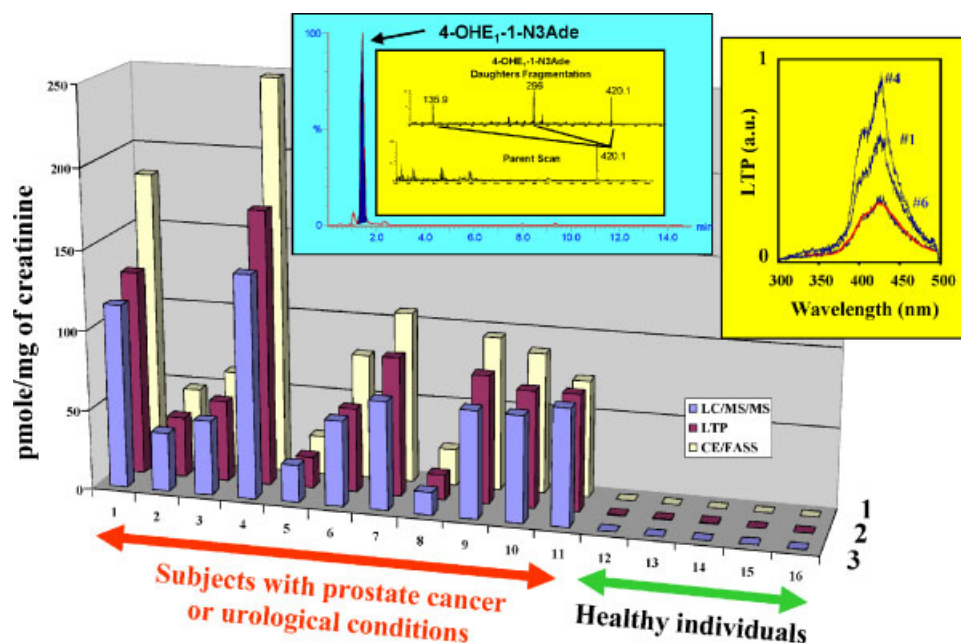


Fig. 2. Identification of the 4-OHE₁-1-N3Ade adduct in human samples from men with prostate cancer or urological conditions and healthy men as control. Right inset: The spectra labeled 1, 4, and 6 refer to individual samples 1, 4, and 6, respectively; the red spectrum is that of the standard. Left inset: Identification of the parent compound and m/z 135.9 and 296 are the fragmentation daughters selected for the unequivocal identification of the adduct.

Finally, LC/MS/MS was used for further validation of the above findings. That is, all samples eluted from the immunoaffinity columns were also analyzed by LC/MS/MS. The amounts of the adducts are reported in row #3 of Figure 2. Only samples #1–11 revealed the presence of the 4-OHE₁-1-N3Ade adducts. Although similar adduct distribution is observed in all samples using the three different methodologies, the relative adduct concentration observed in elutions from immunoaffinity columns was somewhat smaller than that observed by CE/FASS with absorbance detection. This is not surprising, since recovery from a typical column is 70–80% [19,24]. The UPLC chromatogram obtained for sample #11 is shown in the left inset of Figure 2; the main peak near 2 min in the chromatogram corresponds to the 4-OHE₁-1-N3Ade adduct, indicating that the eluent from the immunoaffinity column was relatively pure. The spectrum corresponds to the daughter ions, *m/z* 135.9 and 296.0, which were obtained from fragmentation of the adduct parent ion, *m/z* 420.1. Note that 4-OHE₁-1-N3Ade (and not 4-OHE₂-1-N3Ade) was detected in the urine of all subjects from the urology clinic, but none of the healthy male controls. There were no significant differences between levels from subjects with benign urologic conditions and those with prostate cancer. However, the three highest levels were in a patient with prostate cancer and the two patients with negative biopsies. Thus, it is conceivable that the patients with negative biopsies may harbor undetected prostate cancer, or be at increased risk for prostate cancer. To date, neither man has been diagnosed with prostate cancer at follow-up more than 6 months after the negative biopsy. The sample from the patient with BPH was also among the higher values, indicating that BPH might be related to CEQ-induced DNA damage. However, LC/MS/MS did not reveal the presence of any 4-OHE₁-1-N3Ade adduct in samples #12–16 obtained from healthy individuals, in perfect agreement with the low temperature phosphorescence and CE/FASS studies. Urine samples from several other healthy subjects tested by low-temperature phosphorescence (data not shown) also did not have any detectable 4-OHE₁-1-N3Ade adduct.

CONCLUSIONS

Evidence has been obtained from studies of men with and without prostate cancer that CEQ-derived DNA adducts are formed in humans. We think this is the first demonstration that CEQ-derived DNA adducts are present in urine samples from subjects with prostate cancer. Given the limited sample size, it is difficult to draw conclusions from the lack of significant differences between adduct levels in prostate cancer

cases and men with benign urologic conditions. Larger sample sizes are needed to determine whether the presence of depurinating adducts in human urine samples could be used as a risk factor for prostate cancer. Such studies will also allow us to determine the utility of these biomarkers as surrogate endpoints to investigate the hypothesis that metabolically activated endogenous estrogens are involved in initiating prostate cancer.

ACKNOWLEDGMENTS

This work was supported by a grant from the National Cancer Institute (Program Project Grant 2P01 CA49210-12) and in part by the NIH COBRE award 1 P20 RP15563, and matching support from the State of Kansas. We thank David Matthews, M.D., of Charlotte, NC, for inspiring this study.

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High Concordance of Gene Methylation in Post-Digital Rectal Examination and Post-Biopsy Urine Samples for Prostate Cancer Detection

Craig G. Rogers,* Mark L. Gonzalgo, Gai Yan, Patrick J. Bastian, David Y. Chan, William G. Nelson and Christian P. Pavlovich

From The Brady Urological Institute, Johns Hopkins University School of Medicine, Baltimore, Maryland, and Klinik und Poliklinik für Urologie, Rheinische Friedrich-Wilhelms Universität Bonn, Universitätsklinikum Bonn (PJB), Bonn, Germany

Purpose: We evaluated the concordance between post-digital rectal examination and post-prostate biopsy urine samples using conventional methylation specific polymerase chain reaction analysis of 3 gene promoters in patients with suspected or confirmed prostate cancer.

Materials and Methods: Voided urine specimens were collected from 17 men after 15-second digital rectal examination and again after transrectal ultrasound guided biopsy of the prostate for suspected malignancy or for followup biopsy as part of an expectant management protocol. Urine sediment DNA was isolated and subjected to bisulfite modification. Methylation of GSTP1, EDNRB and APC promoters was determined by conventional methylation specific polymerase chain reaction analysis in post-digital rectal examination and post-biopsy samples, and correlated with clinical information.

Results: Prostate cancer was detected on prostate biopsy in 12 of 17 patients (71%). Promoter methylation was detected in post-digital rectal examination urine specimens for GSTP1 (24%), APC (12%) and EDNRB (66%). Promoter methylation was detected in post-biopsy urine specimens for GSTP1 (18%), APC (18%) and EDNRB (77%). The concordance between post-digital rectal examination and post-biopsy urine samples was 94% for GSTP1 and APC, and 82% for EDNRB. Overall 100% of patients with biopsy proven prostate cancer had at least 1 gene methylated in urine vs 60% of those without evidence of prostate cancer on biopsy.

Conclusions: Gene analysis using conventional methylation specific polymerase chain reaction is a reliable method for detecting abnormal DNA methylation in voided urine samples obtained following digital rectal examination or prostate needle biopsy. The concordance between post-digital rectal examination and post-biopsy urinary samples for promoter methylation is high (82% to 94%), suggesting that urine collected after digital rectal examination may be used for genetic analysis with results similar to those in post-biopsy urine samples.

Key Words: prostate, prostatic neoplasms, biopsy, urine, methylation

Prostate cancer is the most commonly diagnosed cancer and the second leading cause of cancer related death in males older than 40 years in Western industrialized countries. The most common DNA alteration associated with prostate cancer is hypermethylation in the regulatory region of certain genes, particularly in the promoter of the π class GSTP1 gene.¹⁻⁴ Hypermethylation of gene promoter regions is associated with many human cancers.^{2,3} Aberrant GSTP1 methylation has been detected in the urine, ejaculate and prostatic secretions of men with prostate cancer.^{5,6} Analysis of hypermethylation of other gene promoters in combination has demonstrated high sensitivity and specificity for prostate cancer diagnosis.^{7,8}

Prostatic manipulation from sources such as a biopsy needle, TRUS probe or DRE may cause prostatic DNA to appear in urine by the shedding of neoplastic cells or debris into the prostatic ducts and urethra. The specific impact of prostatic

manipulation on the detection of DNA promoter hypermethylation in the urine is unclear because to our knowledge there are no studies comparing urine obtained before and after prostatic manipulation in identical patients. We hypothesized that voided urine specimens from patients with prostate cancer would be more likely to have detectable DNA promoter hypermethylation immediately after prostate manipulation by TRUS guided needle biopsy than after DRE.

We compared voided urine samples obtained after extended 15-second DRE with voided urine samples obtained after TRUS guided needle prostate biopsy from patients with suspected or confirmed prostate cancer. We used conventional MSP analysis to examine the hypermethylation status of the 3 gene promoters GSTP1, APC⁹ and EDNRB. These loci were chosen because of their high frequency of methylation in prostate cancer specimens.⁷ Methylation analysis at multiple genes has also been shown to have diagnostic and prognostic value for prostate cancer.^{10,11}

MATERIALS AND METHODS

Ten men undergoing prostate biopsy for suspected prostate cancer and 7 with previously diagnosed prostate cancer un-

Submitted for publication October 31, 2005.

Study received Institutional Review Board approval.

* Correspondence: James Buchanan Brady Urological Institute, Johns Hopkins Hospital, Baltimore, Maryland 21287 (telephone: 410-955-4494; FAX: 410-955-0833; e-mail: crogers6@jhmi.edu).

dergoing followup biopsy as part of an expectant management protocol were enrolled in the study. Approval was obtained from our Institutional Review Board before initiating the study and all patients provided written informed consent. Voided urine specimens (10 to 100 cc) were prospectively collected from 17 men with a mean age of 63.5 years immediately following 15-second DRE and again after transrectal ultrasound guided prostate biopsy during a single office visit. Molecular urinalysis of promoter methylation was performed by an investigator blinded to biopsy results.

Voided urine specimens were centrifuged for 10 minutes at $1,000 \times$ gravity to isolate cellular material and sediment. Total DNA was extracted from the urine pellet using a QIAamp® Viral RNA Mini Kit. The average DNA concentration yielded was approximately 100 ng/ μ l (range 68 to 150) with an average volume of approximately 80 μ l. DNA was then subjected to sodium bisulfite modification using a CpGenome™ Universal DNA Modification Kit. Concurrently modified were 1 μ g universally M DNA (Chemicon, Temecula, California) and 1 μ g genomic DNA from human male white blood cells (EMD Biosciences, San Diego, California). MSP was used to detect U and M alleles in each sample.¹² MSP was performed using 2 primer pairs, including 1 that detected U alleles and 1 that detected densely M alleles.^{7,13,14}

Table 1 lists the U and M specific primers used in PCR. PCR was performed with 3.0 μ l bisulfite modified DNA template in a 25 μ l reaction mixture containing 2.5 μ l $10 \times$ GeneAmp® reaction buffer II, 200 μ M of each deoxynucleoside triphosphate, $MgCl_2$ at the concentrations indicated, 0.25 μ M of each primer and 1.25 U AmpliTaq Gold® polymerase (table 1). PCR conditions were initial denaturation at 95°C for 10 minutes, followed by 40 to 50 cycles of denaturation at 95°C for 1 minute, annealing at the corresponding annealing temperature for 30 seconds, extension at 72°C for 1 minute and final extension at 72°C for 7 minutes (table 1). PCR products were electrophoresed on 2% agarose gels and visualized with ethidium bromide staining under ultraviolet illumination. Each PCR reaction contained a water blank, a positive control (universally M DNA) and a negative control (white blood cell DNA). All urine samples were processed and analyzed in blinded fashion. Methylation status was then correlated with biopsy results and clinical information, and concordance was determined between post-DRE and post-biopsy urine samples.

Promoter methylation was analyzed in urine specimens after DRE and prostate biopsy to assess the concordance between matched pairs of post-DRE and post-biopsy urine specimens. Sensitivity was calculated to detect methylation

changes using primer sets separately and in combination. The concordance of hypermethylation results was determined for matched post-DRE and post-biopsy urine pairs, and an overall concordance rate was determined for each gene promoter assessed.

RESULTS

All urine samples yielded amplifiable DNA for most genes in our panel. The pattern of hypermethylation for our 3 gene panel was compared between post-DRE and corresponding post-biopsy urine samples. Table 2 shows clinicopathological and gene promoter methylation detection data on the 17 study patients. Prostate cancer was detected on prostate biopsy in 12 of 17 patients (71%). No cancer was identified on prostate biopsy in 5 men (29%). All cases of prostate cancer were clinical stage T1c with Gleason 6 disease on biopsy except in 1 of Gleason 7, clinical stage T2b disease. Four men with prostate cancer underwent RRP. RRP Gleason scores were identical to biopsy Gleason scores in all 4 patients.

We observed hypermethylation of the GSTP1 promoter in voided urine specimens of 4 of the 17 patients (24%) after DRE and in 3 (18%) after prostate biopsy. The frequency of promoter hypermethylation of APC was 2 of 17 patients (12%) for post-DRE urine samples compared to 3 of 17 (18%) for post-biopsy urine samples. The frequency of promoter hypermethylation of EDNRB was 8 of 12 informative post-DRE specimens (66%) compared to 10 of 13 informative post-biopsy urine samples (77%). The concordance between paired post-DRE and post-biopsy urine samples was 94% for GSTP1 and APC, and 82% for EDNRB.

The figure shows representative MSP analysis of paired post-DRE and post-biopsy urine specimens to evaluate promoter hypermethylation. Universally M DNA showed no band in the U lane and a strong band in the M lane, and vice versa for white blood cell DNA. Cases that demonstrated no appropriate PCR product in the U or M lane were classified as NI, possibly indicating an insufficient number of alleles amplifiable by the corresponding primers. Concordance for GSTP1 and APC promoter hypermethylation was seen in all paired post-DRE and post-biopsy samples (see figure). Concordance of hypermethylation at all 3 promoters was observed in certain post-DRE and corresponding post-biopsy urine specimens, including specimens 219, 227 and 230. Concordance between post-DRE and corresponding post-biopsy urine samples for EDNRB promoter hypermethylation was not present for specimen 213 (see figure). The post-DRE urine sample for patient 216 was NI for EDNRB.

TABLE 1. Primers used to amplify bisulfite converted DNA at GSTP1, APC and EDNRB promoter regions

Gene (specific primer)	5'-3' Primer		Amplicon Size (bp)	Annealing Temperature (°C)	$MgCl_2$ (mM)
	Forward	Reverse			
GSTP1:					
U	GATGTTTGGGGTGTAGTGGTTGTT	CCACCCCAATACTAAATCACAACA	97	62	3.0
M	TTCGGGGTGTAGCGGTCGTC	GCCCCAATACTAAATCAGCAGC	91	62	3.0
APC:					
U	GTGTTTATTGTGGAGTGTGGGTT	CCAATCAACAACTCCCAACAA	108	60	3.5
M	TATTGCGGAGTGCGGGTC	TCGACGAACCTCCGACGA	98	62	2.5
EDNRB:					
U	GGGTATTAGGAAGGAGTTTGTATTGTG	CACAACCTCTTACCAATATCCACA	111	62	3.5
M	GGTACGCGGGGAAGAAAAATAGTTG	ATACCGCCCGCAACCTCTTCG	149	65	2.0

TABLE 2. Clinicopathological characteristics and promoter hypermethylation detection status for *GSTP1*, *APC* and *EDNRB* in 17 study patients

Pt No.—Age	PSA (μg/ml)	DRE	Clinical Stage	Gleason	RRP Stage	Post-DRE/Post-Biopsy Urine		
						GSTP1	APC	EDNRB
Neg biopsy								
218—65	8.8	Neg	N/A	N/A	N/A	U/U	U/U	NI/U
219—60	11.3	Neg	N/A	N/A	N/A	M/M*	M/M*	M/M*
227—71	4.8	Neg	N/A	N/A	N/A	U/U	U/U	M/M
230—63	7.2	Pos	N/A	N/A	N/A	U/U	U/U	M/M
238—46	4.7	Pos	N/A	N/A	N/A	U/U	U/U	U/U
Pos biopsy								
228—64	6.1	Neg	T1c	6	N/A	U/U	U/U	M/M
226—58	1.2	Neg	T1c	6	N/A	U/U	U/U	M/M
60—72	0.4	Neg	T1c	6	N/A	U/U	U/U	M/M
105—52	12	Neg	T1c	6	T2NxMx	U/U	U/U	U/M*
176—59	4.4	Neg	T1c	6	T2cNxMx	U/U	U/U	NI/NI
213—59	5.9	Pos	T2b	4+3	T3aN0Mx	M/M	U/U	U/M*
214—67	3.5	Pos	T1c	6	N/A	M/U*	U/M*	U/U
215—62	5	Pos	T1c	6	N/A	U/U	U/U	M/NI
216—72	9.6	Pos	T1c	6	N/A	M/M*	M/M*	NI/M
217—72	5.4	Pos	T1c	6	Radiation	U/U	U/U	NI/NI
225—68	4.9	Pos	T1c	6	T2N0Mx	U/U	U/U	NI/NI
232—69	7.8	Pos	T1c	6	N/A	U/U	U/U	M/M

* Discordant urine pairs.

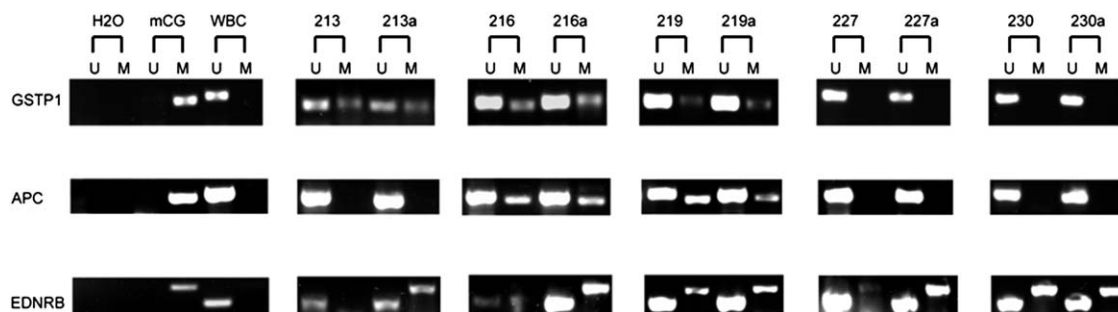
Hypermethylation of the *GSTP1* promoter was observed in 3 of 12 patients (25% sensitivity) with biopsy confirmed adenocarcinoma of the prostate and in 1 of 5 (20%) with no evidence of cancer on biopsy. Hypermethylation of the *APC* promoter was observed in 2 of 12 patients (17% sensitivity) with biopsy confirmed adenocarcinoma of the prostate and in 1 of 5 (20%) with no evidence of cancer on biopsy. Hypermethylation of the *EDNRB* promoter was observed in 6 of 7 informative cases (85% sensitivity) of biopsy confirmed adenocarcinoma of the prostate and in 3 of 5 (60%) with no evidence of cancer on biopsy. All 9 informative cases (100%) with prostate cancer showed at least 1 detectable hypermethylated gene promoter in the urine compared to 3 of 5 (60%) without evidence of cancer on biopsy.

DISCUSSION

Prostatic manipulation from sources such as a biopsy needle, transrectal ultrasound probe and/or DRE may cause DNA to appear in urinary tract fluids due to the shedding of neoplastic cells and debris into the prostatic ducts. The impact of prostatic manipulation on the detection of prostate cancer cells in urine has not previously been described in studies comparing detection rates in urine obtained before

and after prostatic manipulation in identical patients with cancer. To our knowledge our study is the first analysis of concordance between post-DRE and post-biopsy urine samples for promoter hypermethylation. We hypothesized that voided urine specimens from patients with prostate cancer would contain a higher amount of neoplastic cellular material for DNA analysis immediately after TRUS biopsy compared with a voided urine specimen after DRE and, hence, a higher rate of detection of promoter methylation. However, the concordance between post-DRE and post-biopsy urine samples for promoter hypermethylation in our study was high, suggesting that urine collected after DRE may be used for molecular urinalysis with results similar to those of urine samples obtained after more invasive techniques, such as prostate biopsy. Molecular analysis of urine obtained after prostate biopsy could potentially have a limited role for identifying patients with a false-negative biopsy who must undergo repeat biopsy. Post-DRE urine collection is more practical and less invasive than collection after prostate biopsy and it can be applied in a broader group of patients for prostate cancer detection, such as those undergoing routine prostate cancer screening.

Previous studies have shown the efficacy of *GSTP1* promoter hypermethylation for diagnosing prostate cancer in



Representative MSP analysis of paired post-DRE and post-biopsy (α) urine specimens for *GSTP1*, *APC* and *EDNRB* promoter hypermethylation. Prostate cancer was confirmed on prostate biopsy in patients 213 and 216. Prostate biopsy was negative in other patients. PCR product generation indicated U or M alleles. *H2O*, water blank PCR reaction contamination control. *mCG*, universally M DNA positive methylation control. *WBC*, white blood cell DNA negative methylation control.

various bodily fluids with sensitivity approaching 75%.^{6,13,15} We also included other gene promoters in our panel because the sensitivity and specificity of prostate cancer diagnosis in bodily fluids, such as urine, ejaculate and prostate secretions, and biopsy specimens has been shown to be as high as 92% to 100% when several markers are used in combination.^{7,11,15} The 3 genes chosen for our study panel (GSTP1, APC and EDNRB) have cancer and prostate cancer specific hypermethylation, and known biological significance. The diagnostic coverage of our 3 gene methylation panel for diagnosing prostate cancer in voided urine specimens was 100%.

Prostatic manipulation, ie massage, biopsy, etc, may increase the detection of prostate cancer DNA in voided urine from men harboring prostate cancer. Cairns et al noted GSTP1 hypermethylation in normally voided urine of 27% of men with early stage prostate cancer.¹⁶ After 1 minute of prostatic massage Goessl et al were able to detect GSTP1 hypermethylation in 68% of men with early stage prostate cancer.¹⁷ Gonzalgo et al used MSP to examine urine collected after prostate biopsy.¹³ They detected GSTP1 hypermethylation in 58% of patients with biopsy proven prostate cancer and in 33% of patients without prostate cancer or PIN. Gonzalgo et al also analyzed prostatic secretions from RRP specimens and detected GSTP1 hypermethylation in 86% of patients using a combinatorial MSP approach,¹³ which may represent close to the maximal sensitivity of this assay for detecting hypermethylation of this allele in patients with prostate cancer.¹⁶ In the current study DRE was performed for approximately 15 seconds per patient, which may be longer than typical diagnostic DRE. However, it is shorter than a typical prostatic massage of approximately 30 seconds to 1 minute. Our findings suggest that even with modest prostatic manipulation, such as may be performed by 15-second DRE, material of diagnostic value is shed into the prostatic urethra and into subsequently voided urine. Prostatic manipulation by DRE, biopsy, etc may cause spastic contractions of the smooth muscles of the prostate, which can cause nucleic acids and proteins to be trapped inside prostatic acini. Subsequent urination and smooth muscle relaxation may allow the evacuation of these compartments, thus, increasing prostate cancer DNA detection.

A limitation of our study is its small patient population. In addition, the prevalence of prostate cancer in our small cohort was high and it does not represent a typical screening population because we included patients with known prostate cancer who were on an expectant management program.¹⁸ We chose to include these patients because they routinely undergo prostate biopsy and already have a known cancer diagnosis, making them a reliable source for obtaining matched post-DRE and post-biopsy urine specimens for our proof of principle study. We emphasize that our current goal was to assess the concordance between matched post-DRE and post-biopsy urine samples, and so we did not design this study to optimize sensitivity and specificity since high sensitivity and specificity have already been demonstrated in other studies using MSP analysis. However, additional studies including patients with benign prostatic conditions and nonprostatic malignancies are warranted. The high proportion of patients on an expectant management protocol may actually have contributed to the low detection rate of prostate cancer in our study because these patients had low volume cancers on biopsy. Prostate cancer

detection could have potentially been improved in our study by increasing the number and volume of urine samples, and by performing more vigorous prostatic massage. It is unclear to what extent the TRUS probe influences prostatic manipulation during prostate biopsy and the subsequent shedding of cellular debris into urine. We did not analyze urine specimens after TRUS only without biopsy because this additional test would have limited the amount of urine available for analysis for each test and it is rarely clinically indicated. The detection of promoter hypermethylation in our study could theoretically have been biased in favor of post-biopsy urine samples since these patients underwent serial prostatic manipulation (DRE followed by biopsy). However, post-DRE urine samples still compared well to post-biopsy samples with a high concordance for promoter methylation between matched samples.

CONCLUSIONS

Our data suggest that voided urine samples obtained after DRE or after prostate biopsy contain similar epigenetic molecular information. The concordance between post-DRE and post-biopsy urine samples for promoter methylation is high (82% to 94%), suggesting that urine collected after DRE may be used to analyze genetic markers for prostate cancer with results similar to those of post-biopsy urine samples. Validation of this approach in larger, prospective trials and optimization of an appropriate panel of methylation markers may ultimately lead to widespread use of this technology for the early detection and prognostication of prostate cancer.¹⁹

Abbreviations and Acronyms

DRE	=	digital rectal examination
GSTP1	=	glutathione-S-transferase
M	=	methyated
MSP	=	methylation specific PCR
N/A	=	not applicable
NI	=	noninformative
PCR	=	polymerase chain reaction
RRP	=	radical retropubic prostatectomy
TRUS	=	transrectal ultrasound
U	=	unmethyated

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Cytokine Profiling of Prostatic Fluid From Cancerous Prostate Glands Identifies Cytokines Associated With Extent of Tumor and Inflammation

Kazutoshi Fujita,¹ Charles M. Ewing,¹ Lori J. Sokoll,^{1,2} Debra J. Elliott,^{1,2}
Mark Cunningham,¹ Angelo M. De Marzo,^{1,2}
William B. Isaacs,¹ and Christian P. Pavlovich^{1*}

¹*The Brady Urological Institute, The Johns Hopkins Medical Institutions, Baltimore, Maryland*

²*Department of Pathology, The Johns Hopkins Medical Institutions, Baltimore, Maryland*

BACKGROUND. Cytokines are key mediators of inflammation that may relate to prostate cancer initiation and progression, and that may be useful markers of prostatic neoplasia and related inflammation. In order to better understand the relationship between cytokines and prostate cancer, we profiled cytokines in prostatic fluids obtained from cancerous prostate glands and correlated them to both cancer status and inflammatory grade.

METHODS. Prostatic fluid was collected from fresh radical prostatectomy specimens and analyzed by cytokine antibody microarray. For comparison, cases were selected from patients with either minimal or extensive cancer volume on final pathology. Among the cytokines with the greatest difference between the tumor volume groups, eight had their levels quantitated by ELISA. In addition, the grade of prostatic inflammation by neutrophils, macrophages and lymphocytes was scored for each case and examined for correlations with cytokine levels.

RESULTS. Among 174 cytokines analyzed, HGF was the most increased (6.57-fold), and along with IL18Bpa was significantly elevated in patients with extensive disease compared to those with minimal disease. IL17, GITR, and ICAM-1 were elevated in specimens with significant neutrophilic inflammation into gland lumina, and IL18Bpa, IL17, GITR, and ICAM-1 were elevated in specimens with significant lymphocytic inflammation in prostatic stroma.

CONCLUSIONS. Prostatic fluid cytokines were identified that may be useful for early cancer detection and prognostication efforts and for assessment of prostatic inflammation, particularly if they can be found not only in prostatic fluids obtained ex vivo, but in expressed prostatic secretions or urine samples from men with prostates still in situ.

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KEY WORDS: cancer; inflammation; cytokine

INTRODUCTION

Prostate cancer is the most common cancer and the second leading cause of cancer-related death in men over 40 years of age in the United States [1]. The etiology of prostate cancer is not well understood. Chronic infection and inflammation are causes of cancer in the stomach, liver and large intestine. Data from histopathological, molecular histopathological, epidemiological, and genetic epidemiological studies show that chronic inflammation might also be important in prostate carcinogenesis [2]. Proliferative inflammatory atrophy (PIA), where proliferative glandular

This article contains supplementary material, which may be viewed at The Prostate website at <http://www.interscience.wiley.com/jpages/0270-4137/suppmat/index.html>.

K. Fujita and C.M. Ewing contributed equally to this work.

Grant sponsor: NIH/NIDDK; Grant number: 1K23DK071262; Grant sponsor: Department of Defense; Grant number: PC041214; Grant sponsor: NIH/NCI; Grant number: U24 CA115102.

*Correspondence to: Dr. Christian P. Pavlovich, The Brady Urological Institute, A-345, Johns Hopkins Bayview Medical Center, 4940 Eastern Ave., Baltimore, MD 21224. E-mail: cpavlov2@jhmi.edu
Received 12 November 2007; Accepted 1 February 2008

DOI 10.1002/pros.20755

Published online in Wiley InterScience
(www.interscience.wiley.com).

epithelium with the morphological appearance of simple atrophy occurs in association with inflammation, is thought to be a possible precursor to prostate cancer [3]. Chronic and/or acute glandular inflammation is indeed observed in many radical prostatectomy specimens [4].

Cytokines are proteins that are expressed from immune, epithelial, and stromal cells, that can be excreted into the lumina of glands [5,6]. Cells communicate with each other by networks of interrelated cytokines. Cytokines are not only key mediators of inflammation, but may also play important roles in the initiation and progression of prostate cancer. While some cytokine analysis of prostatic fluid from expressed prostatic secretions has been performed [5,6], a comprehensive cataloging of cytokines from the cancerous prostate has not been reported. Such a cytokine profile may provide further insight into the mechanisms of prostate cancer initiation and progression, and may facilitate the exploration of new markers of prostatic neoplasia and inflammation. If chemopreventive strategies aimed at reducing prostatic inflammation are implemented, noninvasive markers of this process would be useful. In this study, we describe the cytokine profile of prostatic fluids obtained from cancerous prostate glands and correlate it to both cancer status and inflammation grade.

MATERIALS AND METHODS

Collection of Samples

Prostatic fluids were collected by squeezing ex vivo prostate glands that were freshly obtained following radical prostatectomy for prostate cancer and collecting drops of fluid from the protruding apical urethral stump. The radical prostatectomy specimens were then submitted for routine formalin fixation, sectioning, and pathologic analysis as per standard protocol [7]. Prostate glands with either minimal prostate cancer (M, $n=20$) or extensive prostate cancer (E, $n=20$) as estimated by tumor volume were chosen for this study. Specimens with minute foci of a maximum tumor area of less than 15 mm^2 were assigned to the M group, and specimens with a maximum tumor area of more than 80 mm^2 were assigned to the E group. The prostatic fluids were kept at -80°C until the cytokine determination experiments. Approval was obtained from our Institutional Review Board before initiating the study and all patients provided written informed consent.

Cytokine Antibody Array

A RaybioTM Human Cytokine Array kit (Raybiotech, Norcross, GA) including 174 cytokines was used

per the manufacturer's recommendations. Briefly, membranes immobilized with capture antibodies were blocked with 5% bovine serum albumin/triethanolamine-buffered saline (TBS) for 1 hr. Membranes were then incubated with prostatic fluid samples [1 ml, in 10-fold dilution with TBS and Complete protease inhibitor cocktail tablets (Roche Diagnostics, Indianapolis, IN)] for 2 hr at room temperature. After extensive washing with TBS/0.1% Tween 20 (3 times, 5 min each) and TBS (twice, 5 min each) to remove unbound cytokines, membranes were incubated with biotin-conjugated anticytokine antibodies. Membranes were washed and then incubated with horseradish peroxidase-conjugated streptavidin (2.5 pg/ml) for 1 hr at room temperature. Unbound materials were washed out with TBS/0.1% Tween 20 and TBS. Finally, the signals were detected by the enhanced chemiluminescence system, followed by additional washing. Spots were visualized using enhanced chemiluminescence (ECL plus Western Blotting System, Amersham Biosciences, Pittsburgh, PA). Membranes were exposed to Kodak X-Omat radiographic film for 1 min per image. Each film was scanned into TIFF Image files, and spots were digitized into densities with Gel-Pro-Analyzer (Media Cybernetics, Bethesda, MD). The densities were exported into Microsoft Excel, and the background intensity was subtracted prior to analysis.

Enzyme-Linked Immunosorbent Assay (ELISA)

Eight cytokines in prostatic fluids were measured by ELISA. A human ELISA kit (Raybiotech) was used to detect hepatocyte growth factor (HGF), interleukin 12p70 (IL12), glucocorticoid-induced tumor necrosis factor receptor (GITR), intercellular adhesion molecule 1 (ICAM-1), and neurotrophin-3 (NT-3). A Quantikine human immunoassay kit (R&D Systems, Minneapolis, MN) was used to detect interleukin 17 (IL17), and epithelial-neutrophil activating peptide (ENA78). DuoSet ELISA development system (R&D Systems) was used to detect interleukin 18 binding protein a (IL18Bpa). Each cytokines was measured based on the manufacturer's recommendations. For examples, to measure HGF, IL12, GITR, ICAM-1, and NT-3 prostatic fluids were diluted accordingly. Samples were added (100 μl /well) in duplicate for incubation for 2.5 hr at room temperature. Biotinylated antibodies were subsequently added (100 μl /well) and incubated for 1 hr at room temperature. Incubation with streptavidin-horseradish-peroxidase (for 15 min) was followed by detection with 3,3',5,5'-tetramethylbenzidine (TMB) for 30 min. The reaction was stopped by the addition of 1.5 M H_2SO_4 . Plates were read using a wavelength of 450 nm on a microplate reader (PHERA star, BMG LABTECH, Durham, NC).

Histological Analysis

Hematoxylin and eosin stained sections were used to assess the inflammatory status of the prostate. For each case, two sections were chosen from right posterior, left posterior, right anterior, and left anterior prostate at apex and middle (eight sections total) and were examined by light microscopy for the presence of neutrophils, macrophages and lymphocytes. An inflammation grade of 1 for neutrophils or macrophages (low-grade inflammation) was assigned to specimens in which neutrophils or macrophages were observed only in prostatic gland lumina, with no epithelial disruption, or in which neutrophils were not observed at all. A grade of 2 (high-grade inflammation) was assigned to specimens in which the gland lumina were filled with immune cells and/or pus and more than 10 neutrophils or macrophages were found in the epithelial lining under 40 \times magnification, or in which these immune cells were found in the interstitium with associated epithelial destruction. A grade of 1 for lymphocytes was assigned to the specimens in which confluent sheets of inflammatory cells with nodule/follicle formation were observed focally or multifocally in the stroma (less than 50% of area), while a grade of 2 was assigned to specimens in which those were observed diffusely in the stroma (more than 50% of area) in at least one section [8].

Data Analysis and Statistics

Positive control signals on each membrane were used to normalize cytokine signal intensities from cytokine antibody arrays. Then, the data were normalized to PSA levels in each prostatic fluid sample to account for differential yields of fluid actually of prostatic origin. Total PSA levels in each prostatic fluid sample were measured by Hybritech PSA assay on the Beckman Coulter Access Immunoassay System

(Beckman Coulter, Inc., Fullerton, CA). The normalized intensity value of cytokines in each group (M or E) was converted into the relative n-fold change between groups.

Data from ELISA in prostatic fluids were also normalized to the average PSA levels in each prostatic fluid sample. Data from prostatic fluids were analyzed as categorized by tumor volume (M and E), Gleason score (6 and ≥ 7), or inflammation grade (1 or 2).

Statistical analyses were done using GraphPad Prism 4.0 for Windows. Mann–Whitney tests were used to analyze the difference of two categories. Chi-square tests were used to analyze the correlations between tumor volume and inflammation grade. Spearman's correlations were used to analyze the correlations of two cytokines and that of cytokines and tissue weights or age. Statistical significance was defined as a *P*-value < 0.05 .

RESULTS

Cytokine Profile of Prostatic Fluid by Cytokine Array

The normalized intensity values of cytokines from group E (extensive volume prostate cancer) were divided by those from group M (minimal volume prostate cancer) to calculate the relative n-fold change. The ranked cytokine profile of the relative n-fold change obtained by cytokine array is listed in Table I; for a comprehensive listing see Supplementary Table. Among 174 cytokines analyzed, HGF was the most increased cytokine in group E (6.57-fold).

Correlation of HGF and IL18BP α With Cancer Status by ELISA

Among the cytokines with the greatest difference between groups E and M, we selected eight cytokines for further study (HGF, IL18BP α , ICAM-1, IL17, NT-3, IL12, GITR, and ENA78) and confirmed their levels in

TABLE I. Cytokine Profile of Prostatic Fluid

Cytokine	Ratio (Ext/Min)	Average signal of Ext group (SD)	Average signal of Min group (SD)
HGF	6.57	118.24 (164.50)	18 (14.90)
IL18BP α	2.58	4.37 (6.67)	1.69 (2.60)
ICAM-1	2.41	53.34 (68.50)	22.15 (23.09)
IL17	2.34	1.74 (2.78)	0.74 (1.15)
NT3	2.32	1.79 (2.38)	0.77 (1.31)
IL12p70	2.32	4.41 (5.92)	1.90 (1.48)
GITR	1.99	3.80 (4.56)	1.91 (1.69)
ENA78	1.74	20.93 (28.61)	11.99 (18.71)

Complete cytokine profile shown in Supplementary Table.

Ext, extensive prostate cancer, Min, minimal prostate cancer.

prostatic fluids quantitatively by ELISA. Each of these cytokines was elevated in group E; the HGF and IL18Bpa elevations were statistically significant compared to group M (Fig. 1). In an analysis based on Gleason score, only IL18Bpa was significantly elevated in specimens with high Gleason grade (≥ 7). Strong

correlations were noted between some cytokines, especially between ICAM-1 and GITR (Spearman's correlation coefficient $r = 0.820$), ICAM-1 and ENA78 ($r = 0.782$), and NT3 and GITR ($r = 0.782$) (Table II). No correlation was found between each cytokine and specimen weight. Weak correlations were found

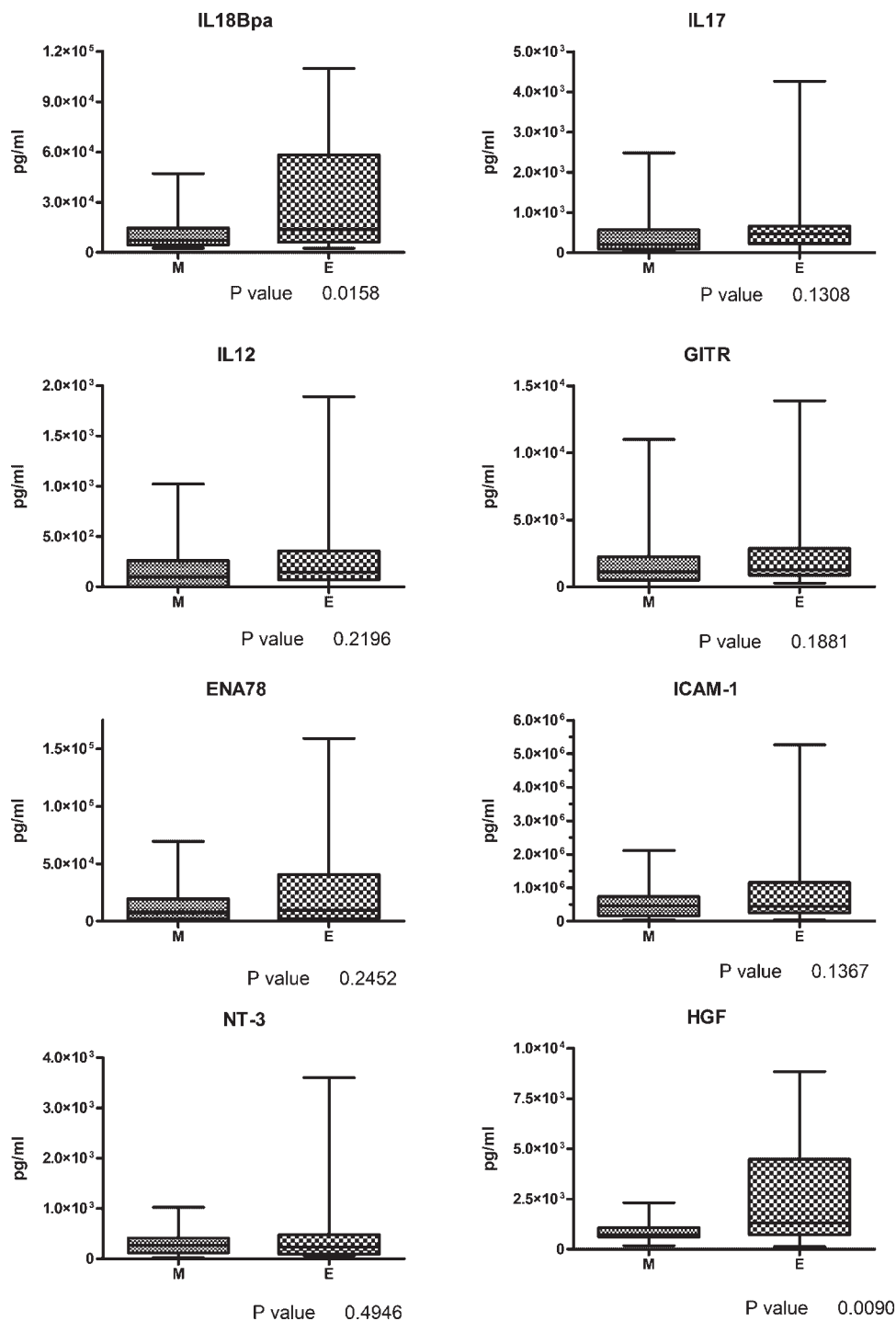


Fig. 1. Correlation of cytokines with cancer status. Each cytokine level measured by ELISA was analyzed stratified by cancer status. The HGF and IL18Bpa elevations of group E were statistically significant compared to group M [M: minimal prostate cancer (n = 20), E: extensive prostate cancer (n = 20)].

TABLE II. Correlations Between Eight Cytokines

	IL18Bpa	IL17	IL12p70	GITR	ENA78	ICAM-1	NT-3	HGF
IL18Bpa								
IL17	0.2585 (0.1072)		0.2992 (0.0643)	0.5417 (0.0004)	0.339 (0.0324)	0.6246 (<0.0001)	0.4507 (0.0035)	0.3732 (0.0177)
IL12p70	0.2992 (0.0643)	0.6486 (<0.0001)		0.4986 (0.0012)	0.4026 (0.01)	0.4829 (0.0016)	0.4865 (0.0015)	0.2568 (0.1096)
GITR	0.5417 (0.0004)	0.4986 (0.0012)	0.6175 (<0.0001)		0.5767 (0.0001)	0.5227 (0.0006)	0.5832 (<0.0001)	0.3227 (0.0451)
ENA78	0.339 (0.0324)	0.4026 (0.01)	0.6175 (<0.0001)	0.6854 (<0.0001)		0.8203 (<0.0001)	0.7816 (<0.0001)	0.3967 (0.0124)
ICAM-1	0.6246 (<0.0001)	0.4829 (0.0016)	0.5767 (0.0001)	0.6854 (<0.0001)	0.7822 (<0.0001)		0.5548 (0.0002)	0.3946 (0.0118)
NT-3	0.4507 (0.0035)	0.4865 (0.0015)	0.5227 (0.0006)	0.8203 (<0.0001)	0.5548 (0.0002)	0.6576 (<0.0001)		0.4927 (0.0012)
HGF	0.3732 (0.0177)	0.2568 (0.1096)	0.3227 (0.0451)	0.7816 (<0.0001)	0.3946 (0.0118)	0.4927 (0.0012)	0.2874 (0.0721)	

The value listed is the Spearman's correlation coefficient, and that in parentheses is the *P*-value.

between increasing age and IL12 ($r=0.3480$) and increasing age and NT3 ($r=0.4001$).

Relationship Between Cytokine Levels and Prostatic Inflammation

Routine histochemical analysis demonstrated that neutrophils and macrophages were present in prostatic glandular lumina (grade 1 inflammation, Fig. 2) and in the lining of the prostate epithelium (grade 2 inflammation). Isolated lymphocytes aggregated in the stroma surrounding ducts (grade 1 cases) and lymphoid follicles were occasionally noted (grade 2). There was no statistical correlation between the inflammation grade by each immune cell type assessed (neutrophil, macrophage, and lymphocyte) and tumor volume (M and E) (Chi-square test). Data pertaining to eight cytokines found in prostatic fluids were analyzed according to inflammation grade in the radical prostatectomy specimens. In cases stratified by neutrophil inflammation, IL17, GITR, and ICAM-1 were significantly associated with increasing (grade 2) inflammation ($P < 0.05$), and ENA78 ($P = 0.0594$) and NT-3 ($P = 0.0554$) levels were close to reaching statistical significance (Fig. 3). In cases stratified by macrophage inflammation, none of these cytokines was significantly elevated in grade 2 versus grade 1 infiltrates (Fig. 4). In cases stratified by lymphocyte inflammation, IL18Bpa, IL17, GITR and ICAM-1 were significantly elevated in grade 2 lymphocytic infiltration ($P < 0.05$) (Fig. 5).

DISCUSSION

The prostate gland secretes many substances, including citric acid, polyamines, zinc, and cytokines. Cytokines are secreted from lymphocytes, macrophages, and mast cells, and also from prostatic epithelial and stromal cells [9–11]. Recently, cytokines have been shown to play important roles in prostatic inflammation, carcinogenesis, and cancer progression [9,12,13]. In this study, we for the first time describe the cytokine profile of prostatic fluid from cancerous prostates. A better knowledge of the cytokines present in prostate fluid may aid in understanding the implications of the prostatic cytokine network on inflammation, carcinogenesis, and prostate cancer progression, and may lead to novel cancer detection strategies.

We initially studied prostatic cytokines by array, and catalogued the most prevalent cytokines noted from fluids obtained from prostate specimens with extensive cancer as compared to those from prostates with minimal cancer. Among the most up-regulated cytokines in cases with extensive disease, we selected HGF, IL18Bpa, ICAM-1, IL17, NT-3, IL12, GITR, and ENA78, for more quantitative assessment by ELISA. These cytokines were selected from the groups of

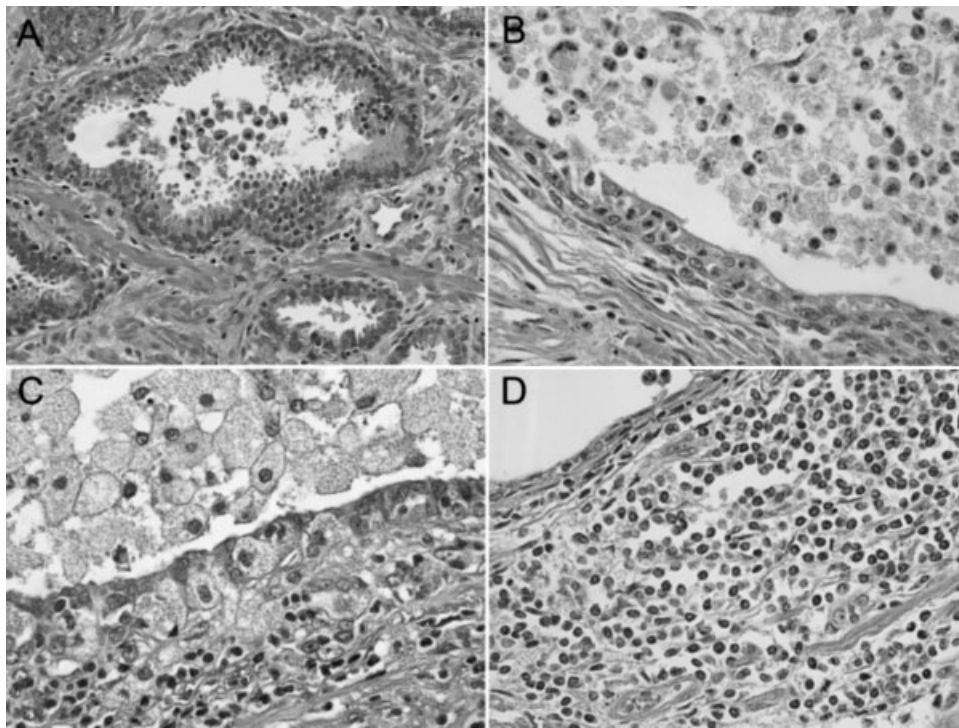


Fig. 2. Inflammation of prostate by neutrophils, macrophages or lymphocytes. **A:** Some neutrophils or macrophages were observed only in gland lumina, with no epithelial destruction. **B:** A gland lumen filled with neutrophils and pus, with neutrophils noted within the epithelial lining (inflammation grade 2). **C:** A gland lumen filled with macrophages and pus, with macrophages noted within the epithelial lining (inflammation grade 2). **D:** In grade 2 lymphocytic inflammation, confluent sheets of inflammatory cells with nodule/follicle formation were observed diffusely in the stroma (more than 50% of the area) in at least one section.

cytokines that were elevated because of their known roles in cancer and inflammation-related pathways.

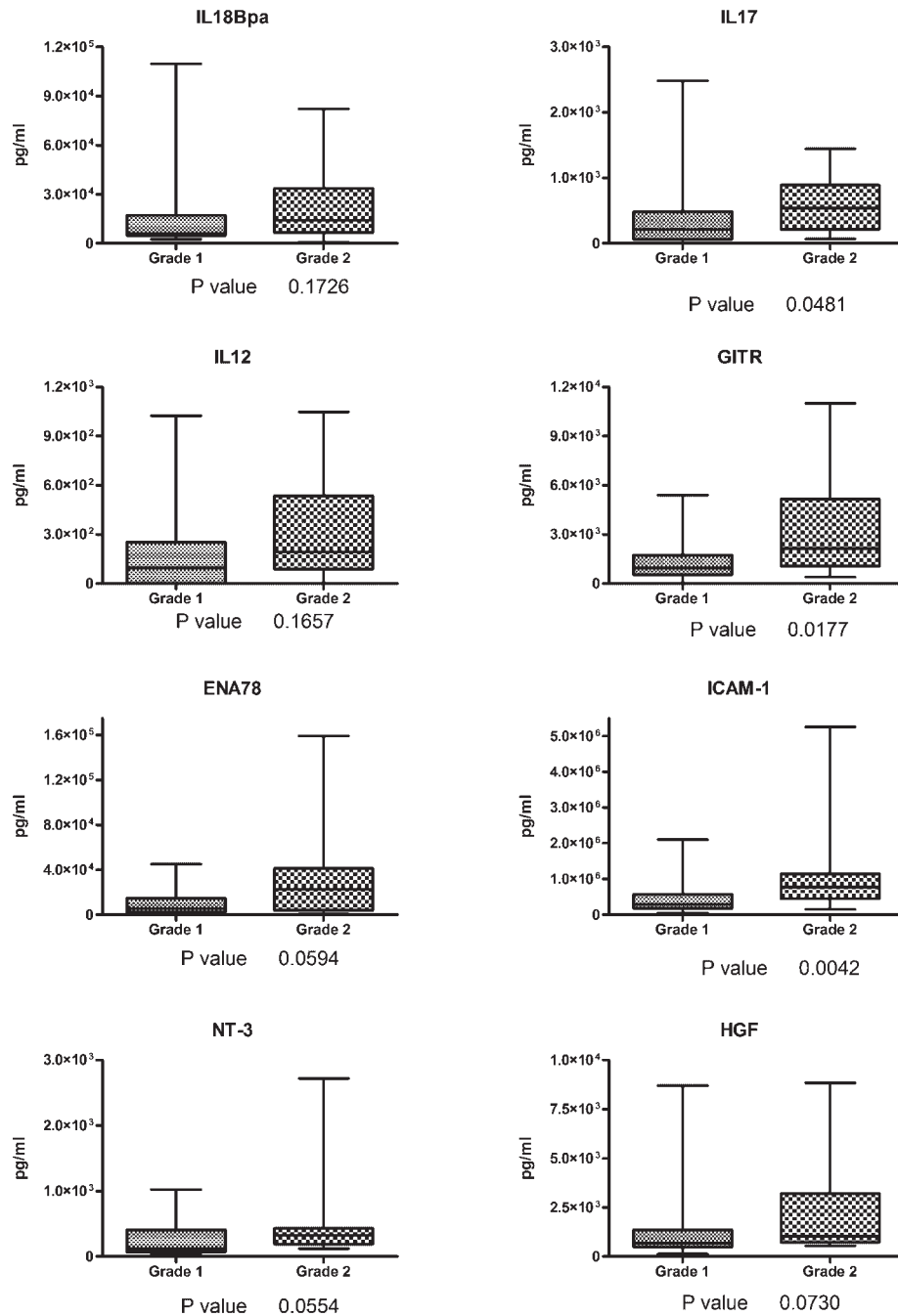
HGF has been shown to be important in prostate cancer progression, invasion and metastasis [14]. IL18Bpa and IL12 are involved in the Th1 immune response [15,16]. IL-12 is the major cytokine responsible for the differentiation of T helper 1 cells, which are in turn potent producers of IFN- γ [15]. IL18Bpa is a potent inhibitor of IL-18, which is a central player in inflammation and in the immune response, and which has antineoplastic properties. ICAM-1 is expressed by leukocytes, epithelial cells, endothelial cells and tumor, stimulates neovascularization [17], and is elevated in the serum of patients with cancer [18]. IL-17 is a pro-inflammatory cytokine, that plays a crucial role in the development of autoimmunity and allergic reactions, and the expression of IL17 from Th17 is stimulated by IL23, which promotes tumor incidence and growth [19]. NT-3 is a member of the neurotrophins; it is expressed by prostate epithelial cells and stromal cells from prostates with cancer, but not by benign prostatic tissue [10]. GITR is expressed by T regulatory cells (Treg) as well as activated T cells and NK cells. ENA-78 produced by monocytes, macrophages, fibroblasts, endothelial cells, and several types of epithelial cells is a member of the CXC family of

chemokines, and acts as a potent chemoattractant and activator of neutrophil function as well as an angiogenic factor in cancer [20,21].

In the cytokine antibody array portion of our study, HGF in prostatic fluid was the cytokine most increased in extensive disease cases, a finding that was confirmed statistically by ELISA. HGF, which can be derived from a variety of tissues, is known to be elevated in the serum of men with metastatic prostate cancer [22]. In the prostate, stromal cells secrete HGF, which acts locally on prostate epithelial cells expressing its receptor, the tyrosine kinase c-Met. Prostate cancer can also express HGF via stimulation by IL-1 β , PDGF, bFGF, VEGF, and EGF derived from stromal cells [23]. The intracellular cascade that ensues secondary to c-Met phosphorylation appears to be responsible for most of the effects of HGF, including its pro-mitogenic and antiapoptotic properties, and its effects on developmental cell migration. Alterations of HGF or c-Met levels can affect these and other biological pathways associated with cancer progression [14].

While prostatic fluid HGF and IL-18Bpa levels were related to tumor volume, prostatic fluid IL-18Bpa, IL17, GITR, and ICAM-1 levels were correlated with inflammation. These results indicate that the above cytokines may be regulated or released by specific immune cells

Neutrophil Inflammation



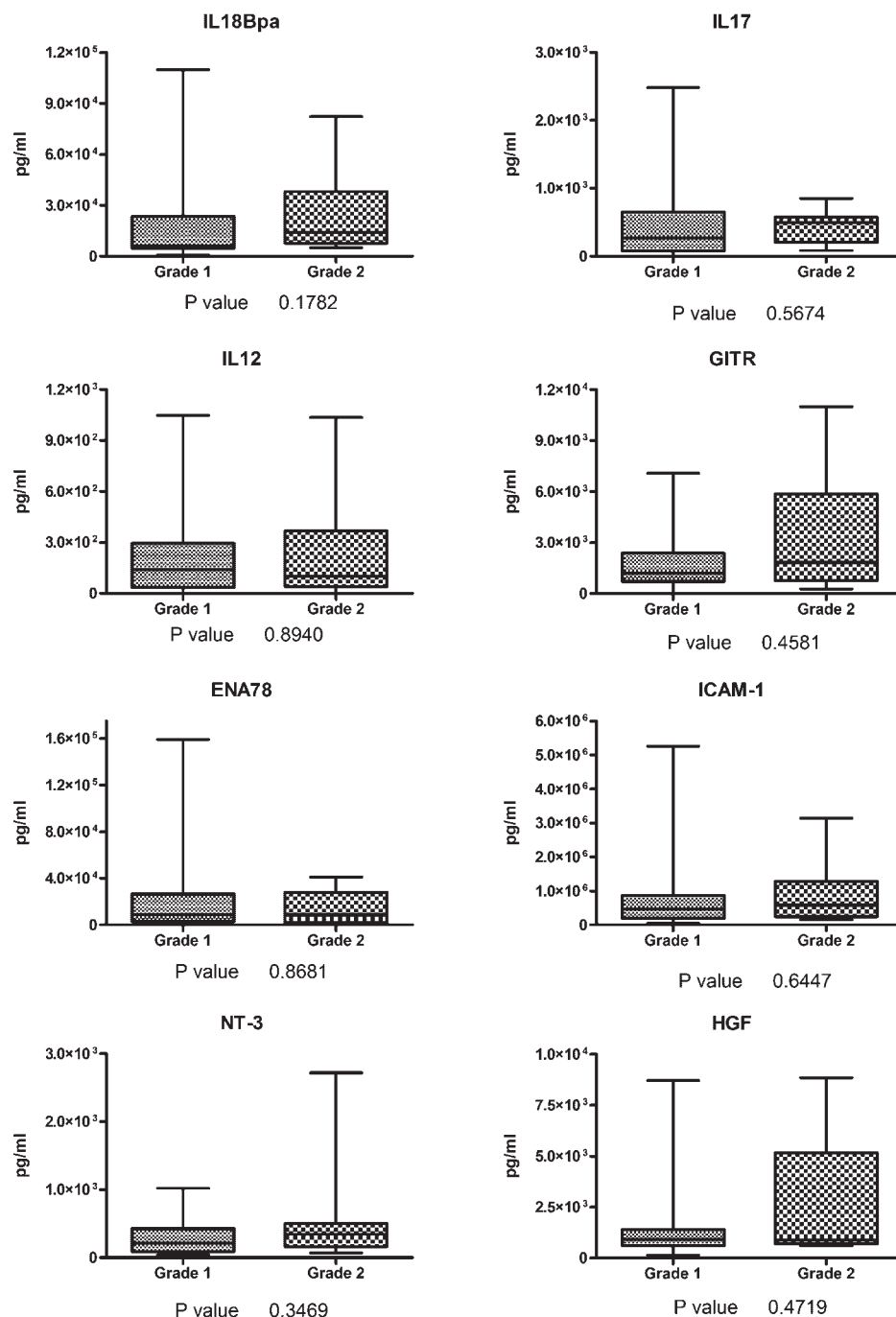
Grade 1: n=22, Grade 2: n=15

Fig. 3. Relationship between cytokine levels and neutrophil inflammation. IL17, GTR, and ICAM-1 were significantly associated with grade 2 inflammation ($P < 0.05$).

in the gland lumina (neutrophils) or in the epithelial lining or stroma (lymphocytes). In fact, IL17 is expressed by Th17, a distinct T cell subset that stimulates the production of cytokines that attract neutrophils to

the site of inflammation [24]. Neutrophils use ICAM-1 on epithelial cells to migrate across the epithelial lining [25], and ICAM-1 is also one of the cytokines induced by IL17 [24].

Macrophage Inflammation



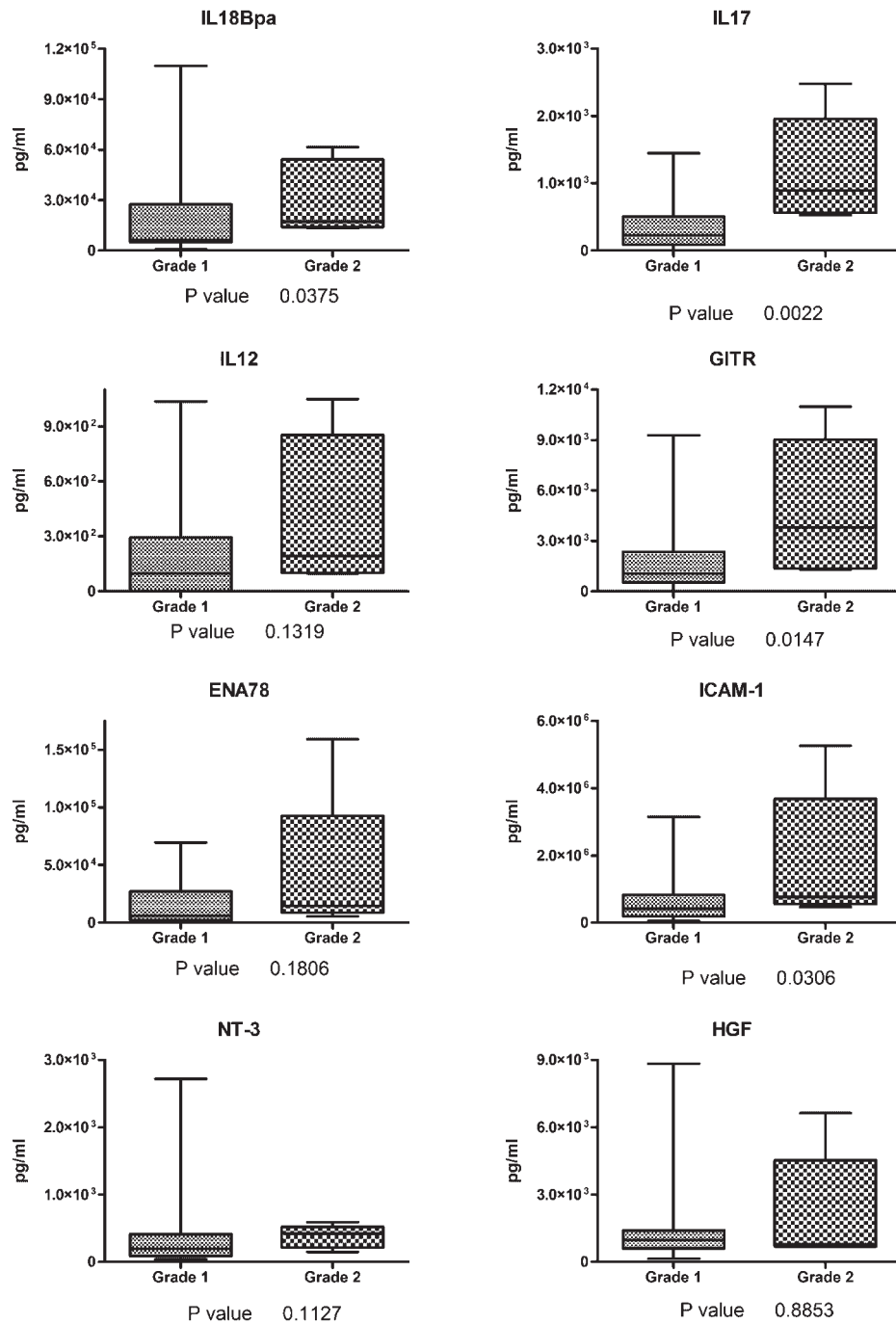
Grade 1: n=29, Grade 2: n=8

Fig. 4. Relationship between cytokine levels and macrophage inflammation. No cytokine was significantly elevated in grade 2 versus grade 1 inflammation.

Among these cytokines, IL18Bpa and GITR may be new markers of prostatic inflammation that is associated with cancer initiation or progression. Importantly, IL18Bpa was correlated with both cancer and inflammation status in our study.

IL18 plays an important role in host defenses against various infectious microbes, but overproduction of IL18 causes autoimmune diseases and inflammatory tissue damage [26]. The excretion of IL18Bpa from monocytes and NK cells is induced by IL12 and

Lymphocyte Inflammation



Grade 1: n=31, Grade 2: n=6

Fig. 5. Relationship between cytokine levels and lymphocyte inflammation. IL18Bpa, IL17, GITR, and ICAM-1 were significantly elevated in grade 2 lymphocytic inflammation ($P < 0.05$).

interferon gamma, and IL18Bpa limits the inflammatory response induced by IL18 [27]. IL18Bpa is also secreted by colon cancer cell lines after interferon gamma stimulation [28], which suggests that prostate

cancer cells themselves may also secrete IL18Bpa upon stimulation by lymphocyte-derived cytokines with the background of inflammation. Since IL18Bpa inhibits the antitumor cytokine IL-18, the finding of IL18Bpa in

malignant prostates suggests an attempt by the cancer to escape immune surveillance and may be correlated with poor prognosis.

GITR has been shown to co-stimulate T cells and abrogate suppression of Treg [29], and to diminish NK cell antitumor immunity [30]. GITR also correlates with neutrophilic infiltration. In GITR^{-/-} mice, neutrophil infiltration into arthritic areas was significantly less than in GITR^{+/+} mice [31]. Whereas GITR-expressing Treg help limit collateral tissue damage caused by vigorous antimicrobial immune response in normal tissues [32], Treg cells are increased in human solid tumors and an increased number of Treg cells correlates with poor prognosis [33]. The increase of GITR induced by the inflammation may be associated with the increase of Treg which suppress the antitumor immunity.

We found strong correlations between the expression levels of certain cytokines: ICAM-1 and GITR, ICAM-1 and ENA78, and GITR and NT-3. ENA-78 strongly attracts neutrophils, and the adhesion of neutrophils to vessel walls or epithelial cells in an area of inflammation occurs via ICAM-1 [34]. It is plausible that GITR-expressing cells, such as regulatory T cells or NK cells, stimulate the expression of NT-3 or ICAM-1 on prostate cancer cells; it may also be that GITR-expressing cells also express ICAM-1 and/or NT-3. Elucidating the reasons for the correlation between these cytokine pairs will require further studies.

A limitation of our study is that we did not assess the cytokine profile of prostatic fluid derived from prostates that were completely benign. The reason for this is that radical prostatectomy (complete removal of the prostate) is not performed on patients without prostate cancer. One consideration was to analyze the cytokine profile of prostatic fluid derived from radical cystoprostatectomy cases in men shown pathologically not to have prostate cancer. However, these men by definition have high grade and/or muscle-invasive bladder cancer neighboring the prostate, which might result in a cytokine profile difficult to discriminate from that associated with urothelial cancer (which can also reside in the prostatic urethra). Rather than selecting such patients, we chose to make our comparisons between cases with very minimal prostate cancer (M) and those with prostate cancers of significant volume (E). Interestingly, one case in the M group was diagnosed with prostate cancer by biopsy, but had no cancer found in the radical prostatectomy specimen despite intensive re-sectioning. While this case cannot be considered a completely negative control for prostate cancer, analysis of prostatic fluids from this case by ELISA did not demonstrate any significant differences in comparison with the average data derived from the other M group cases. In addition,

when we controlled for specimen weight as a surrogate of BPH, we did not note any significant differences in cytokine levels across all cases. An advantage to using RRP specimens for the source of prostatic fluids analyzed in this study is that the entire prostate has been carefully examined for cancer and inflammation in a way that is uniquely afforded by having the entire gland available through radical surgery. Although all of the minimal disease cases analyzed (one with exception) have cancer, we are sure that it is a small amount ($<15 \text{ mm}^2$). Thus, we feel that the cytokine profiles we have described delineate important differences between early, small cancers and late, more extensive cancers and regarding the related inflammatory status of the prostate.

CONCLUSIONS

We hope that our cytokine data provide information that is helpful to researchers studying cytokine networks, paracrine stimulation pathways, and oncogenesis in the prostate. HGF and IL18Bpa were elevated in prostatic fluid from patients with extensive prostate cancers. IL17, GITR, ICAM-1, and IL-18Bpa were elevated in prostatic fluid from specimens with neutrophil inflammation in gland lumina, and IL18Bpa, IL17, GITR, ICAM-1 were elevated in fluid from specimens with lymphocytic inflammation in stroma. These and other cytokines may perhaps be useful in early detection and prognostication efforts if they are found not only in prostatic fluid obtained *ex vivo*, but in expressed prostatic secretions or post-DRE urine samples from patients with their prostates *till in situ*.

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Original contribution

Specific detection of prostate cancer cells in urine by multiplex immunofluorescence cytology[☆]

Kazutoshi Fujita MD^{a,b}, Christian P. Pavlovich MD^b, George J. Netto MD^{a,b,c},
Yuko Konishi BS^a, William B. Isaacs PhD^{b,c}, Syed Ali MBBS^a,
Angelo De Marzo MD, PhD^{a,b,c}, Alan K. Meeker PhD^{a,b,*}

^aDepartment of Pathology, The Johns Hopkins Medical Institutions, Baltimore, MD 21231, USA

^bDepartment of Urology, The Johns Hopkins Medical Institutions, Baltimore, MD 21231, USA

^cDepartment of Oncology, The Johns Hopkins Medical Institutions, Baltimore, MD 21231, USA

Received 10 November 2008; revised 7 January 2009; accepted 9 January 2009

Keywords:

Prostate cancer;
Urine cytology;
Biomarkers;
Diagnosis;
Multiplex staining

Summary Prostate cancer biomarkers are enriched in urine after prostatic manipulation, suggesting that whole cells might also be detectable for diagnosis. We tested multiplex staining of urinary sediments as a minimally invasive method to detect prostate cancer. Urine samples were collected from 35 men who had prostatic massage (attentive digital rectal examination) in a urology clinic and from 15 control men without urologic disease and without massage, for a total of 50 specimens (27 cancer-positive cases and 23 cancer-negative cases). LNCaP prostate cancer cells spiked into urine were used for initial marker optimization. Urine sediments were cytospun onto glass slides and stained. Multiplex urine cytology was compared with conventional urine cytology for cancer detection; anti- α -methylacyl-CoA racemase antibody was used as a marker of prostate cancer cells, anti-Nkx3.1 as a marker of prostate epithelial cells, anti-nucleolin as a marker of nucleoli, and 4'-6-diamidino-2-phenylindole to highlight nuclei. Prostate cancer cells were successfully visualized by combined staining for α -methylacyl-CoA racemase, Nkx3.1, and nucleolin. Of the 25 informative cases with biopsy-proven prostate cancer, 9 were diagnosed as suspicious or positive by multiplex immunofluorescence urine cytology, but only 4 were similarly judged by conventional cytology. All cases without cancer were read as negative by both methods. The multiplex cytology sensitivity for cancer detection in informative cases was 36% (9/25), and specificity was 100% (8/8). In conclusion, we have successfully achieved multiple staining for α -methylacyl-CoA racemase, Nkx3.1, nucleolin, and 4'-6-diamidino-2-phenylindole to detect prostate cancer cells in urine. Further refinements in marker selection and technique may increase sensitivity and applicability for prostate cancer diagnosis.

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[☆] This work was supported by the following research funding mechanisms: NIH/NIDDK grant 1K23DK071262 (C. P. Pavlovich), Department of Defense grant W81XWH-05-1-0167 (C. P. Pavlovich), and a Patrick C. Walsh Foundation grant (A. K. Meeker).

* Corresponding author. Division of Genitourinary Pathology, Department of Pathology, The Johns Hopkins Medical Institutions, Baltimore, MD 21231, USA.
E-mail address: ameeker1@jhmi.edu (A. K. Meeker).

1. Introduction

A prostate cancer (PC) diagnosis is suspected when prostate-specific antigen (PSA) and/or digital rectal examination (DRE) findings are abnormal. Prostate biopsy is currently the only way to confirm the diagnosis of clinically localized PC after abnormal PSA and/or DRE, and is not without risks, including local infection, systemic sepsis, and hemorrhage. In addition, prostate biopsy suffers from inherent inadequacies in tissue sampling, resulting in a sensitivity deficit (false negatives); that is, many cancers are missed. Nevertheless, prostate biopsy remains the criterion standard for PC detection and is the basis for preoperative pathologic grading and cancer volume estimation. A noninvasive and definitive test for PC would be most welcome to patients and clinicians, whether by imaging or molecular biofluid analysis. More effective tools to discriminate patients who harbor undetected cancer from those who do not and who harbor clinically significant cancer are needed.

It is known that PC cells are shed into biologic fluids, particularly when the prostate is subjected to physical manipulation, thus creating the potential for their non-invasive detection in either urine or expressed prostatic fluid (EPF) [1,2]. However, unlike bladder cancer, where urine cytology has proven useful in cancer detection and surveillance, past attempts at detecting PC cells in urine by traditional cytology were thwarted by unacceptably low sensitivities, although specificities were consistently high. This sensitivity deficit was due primarily to the low numbers of PC cells present in urine cytology preparations plus difficulty in differentiating malignant prostate cells from the other cells and debris present in Pap stained urine cytology slides [2-6]. This problem became even worse after the widespread use of PSA testing, which contributed to downward stage migration such that most PCs detected in the modern era are much smaller in volume, lower in grade, and probably less likely to shed malignant cells into the urine. Consequently, detecting PC cells via urine cytology has been largely abandoned. Rather than whole cells, recent attention has focused on detecting various molecular markers linked to PC, including proteins, enzymatic activities, coding and noncoding RNA species, and modified DNA (eg, gene promoter region hypermethylation), each displaying various degrees of sensitivity and specificity for PC detection [7-12]. None of these tests has replaced prostate biopsy for diagnosis, though each confers differing levels of diagnostic importance. Indeed, much progress has recently been made in identifying and detecting novel molecular alterations present in PC cells [13,14]. Considering the expanding pool of candidate biomarkers plus current methods for sensitive marker visualization unavailable to previous cytologists, we revisited the cell-based approach to PC detection in urine samples. In this pilot study, we used simultaneous fluorescent costaining of multiple PC biomarkers to allow

the detection of even a few cancer cells if present in the urine. If definitively identified, PC cells shed in urine, in conjunction with biomarkers and improved imaging modalities, may one day supplant the modern 12-core biopsy for actual PC diagnosis.

2. Materials and methods

2.1. Cell culture

LNCaP PC cells and T24 bladder cancer cells were obtained from ATCC and maintained as adherent cultures in RPMI-1640 medium supplemented with 10% fetal bovine serum (plus 1 nmol/L R1881 for LNCaP). To mimic clinical urine samples, we spiked 5×10^3 LNCaP cells into 10 mL of urine freshly obtained from a normal male volunteer.

2.2. Patients and urine specimens

Urine specimens were prospectively collected from 35 men, under an institutional review board–approved protocol. This study cohort comprised 12 men about to undergo diagnostic prostate needle core biopsy for standard indications (abnormal DRE, elevated PSA, or elevated PSA velocity), 1 patient before transurethral resection for symptomatic lower urinary tract symptoms, 2 men on expectant management for known small volume, low-grade PC, and 20 men with known PC referred for a second opinion regarding definitive treatment. Clinicopathologic characteristics of this cohort are listed in Table 1. Ages ranged from 40 to 76 years, and serum PSA levels ranged from 0.6 to 20.5 ng/mL. Before the biopsy, an “attentive” (approximately 30 seconds) DRE was performed, and the first 50 mL of voided urine were collected. An additional 15 control specimens were collected, without prostate massage, from men without suspicion of PC or with no evidence of disease (NED) after definitive treatment of PC (11 male volunteers without standard indications for PC, 3 patients with NED after radical prostatectomy for PC, 1 patient with NED after radiation therapy for PC). All urine specimens were stored refrigerated until processed, which typically occurred within 3 hours of collection.

2.3. Sample preparations

To develop a method for the isolation of intact PC cells from urine and their deposition onto microscope slides suitable for multiplex staining, we used LNCaP PC tissue culture cells added to freshly collected urine to simulate the behavior of PC cells in patient urine specimens. For urine spiked with tissue culture cells and one-half volume of each post-DRE urine sample, the specimens were centrifuged at 800g for 5 minutes, and pellets were suspended in phosphate-buffered saline (PBS) buffer. Cell suspensions

Table 1 Clinicopathologic information on the study cohort

Case no.	Prostate biopsy status	Biopsy Gleason	Clinical stage	Pathol. stage	Standard urine cytology	Molecular urine cytology	Tumor volume	Age	Race	PSA (ng/mL)
1	—	—	—	—	N	N	—	53	W	4.2
2	—	—	—	—	N	N	—	65	W	9.1
3	—	—	—	—	N	N	—	62	W	0.6
4	—	—	—	—	N	N	—	54	W	3.4
5	—	—	—	—	N	N	—	57	W	4.4
6	—	—	—	—	N	N	—	72	W	7.1
7	—	—	—	—	N	N	—	74	W	5.9
8	—	—	—	—	N	N	—	40	W	0.8
9	+	4 + 3	cT1c	pT3bN0	N	N	Ext.	56	W	5.3
10	+	3 + 3	cT1c	pT2	N	N	Mod.	50	B	6.8
11	+	3 + 3	cT1c	pT2	N	N	Mod.	50	B	6.2
12	+	3 + 4	cT1c	pT2cN0	Uninformative	Uninformative	Ext.	45	W	4.5
13	+	3 + 3	cT1c	NA	N	N	Small	57	W	2.4
14	+	3 + 3	cT1c	pT3aN0	N	N	Ext.	54	W	4.9
15	+	3 + 3	cT1c	pT2aN0	N	N	Small	50	W	4.8
16	+	3 + 3	cT1c	NA	N	N	Ext.	45	W	9.6
17	+	3 + 3	cT2a	NA	N	N	Mod.	58	W	6.6
18	+	3 + 3	cT2a	NA	N	N	Ext.	64	W	6.0
19	+	3 + 3	cT1c	NA	N	N	Small	76	W	4.9
20	+	3 + 3	cT1c	NA	N	N	Small	49	W	3.7
21	+	4 + 4	cT2b	NA	N	Cancer	Ext.	76	W	20.5
22	+	3 + 3	cT1c	NA	N	Suspicious	Small	61	W	6.6
23	+	3 + 3	cT1c	pT2	N	Suspicious	Mod.	53	W	2.5
24	+	3 + 3	cT1c	NA	Cancer	Cancer	Mod.	71	W	8.1
25	+	3 + 4	cT1c	NA	N	Cancer	Mod.	60	B	11.3
26	+	3 + 3	cT2b	pT3a	N	Suspicious	Ext.	56	B	5.5
27	+	3 + 3	cT2b	pT2cN0	Suspicious	N	Ext.	59	W	4.8
28	+	3 + 3	cT1c	NA	N	N	Small	54	W	4.6
29	+	3 + 3	cT1a	NA	N	Suspicious	Small	70	W	2.4
30	+	3 + 4	cT1c	pT2	Suspicious	Suspicious	Ext.	59	W	5.2
31	+	3 + 3	cT1c	pT2cN0	N	Uninformative	Mod.	58	W	17.0
32	+	3 + 3	cT1c	NA	N	N	Mod.	68	W	4.4
33	+	3 + 3	cT2b	pT3bN0	Suspicious	N	Ext.	56	W	7.9
34	+	3 + 3	cT1c	NA	N	N	Small	68	W	5.4
35	+	3 + 3	cT1c	pT2cN0	N	Cancer	Small	45	W	6.9

NA indicates not applicable; N, normal; W, white; Ext., extensive; Mod., moderate; B, black.

were then applied to PrepStain Density Reagent (Tripath Imaging, Burlington, NC) and centrifuged at 200g for 2 minutes, and the top layers were removed and centrifuged at 800g for 10 minutes. The pellets were then resuspended in PBS and were applied to charged surface microscope slides by centrifugation in a cytocentrifuge at 1000 rpm for 3 minutes (Shandon Cytospin IV; Thermo Scientific, Waltham, MA). By this procedure, 2 slides were made from each urine sample. On separate areas of the same cytopsin slides, T24 bladder carcinoma cells were deposited, serving as negative controls for antibody staining. While optimizing multiplex staining, several different fixatives were assayed including acid alcohols, acetone, zinc, paraformaldehyde, and phosphate-buffered neutral formalin. Formalin fixation was eventually chosen for use

because it displayed good compatibility for staining with multiple different markers. Slides were kept at 4°C in PBS until used.

2.4. Antibodies and immunostaining

2.4.1. Slide pretreatment

Of the several different target retrieval buffers assessed for heat-induced epitope retrieval before antibody staining, we found that immersion of the slides for 1 minute in 2% Tween-20 detergent followed by steam heating in high pH target retrieval solution (cat. no. S3307; DAKO Cytomation, Carpinteria, CA) for 20 minutes, then PBS with 0.05% Tween 20 for 5 minutes, produced good results for the particular marker combinations used in these studies.

2.4.2. Immunofluorescence

Primary antibodies were incubated overnight at 4°C. The following antibodies were used in these studies: rabbit monoclonal anti- α -methylacyl-CoA racemase (AMACR) (cat. no. 13H4; Zeta, Sierra Madre, CA), rabbit polyclonal anti-Nkx3.1 [15], and mouse monoclonal anti-human telomerase reverse transcriptase (hTERT) antibody (cat. no. NCL-hTERT [Novocastra, Newcastle, UK], shown to be specific for nucleolin rather than hTERT) [16]. For detection of the rabbit polyclonal anti-AMACR and anti-Nkx3.1 antibodies, signal amplification was performed using the Catalyzed Signal Amplification (CSA) detection system (DAKO Cytomation), followed by application of 1:100 diluted streptavidin-Alexa Fluor 488 conjugated fluorescent secondary reagent (cat. no. S-32354; Molecular Probes, Eugene, OR) for 45 minutes at room temperature. For detection of the mouse monoclonal antinucleolin antibody, Alexa Fluor 568 goat antimouse immunoglobulin G (cat. no. A11061, Molecular Probes) was applied at a dilution of 1:100 in PBS for 45 minutes at room temperature. Slides were then stained with the nuclear counter stain 4'-6-diamidino-2-phenylindole (DAPI) (cat. no. D-8417; Sigma Chemicals, St Louis, MO) and mounted with Prolong™ Anti-fade Mounting Media (cat. no. P-7481, Molecular Probes) and coverslipped.

2.5. Evaluation of fluorescently stained slides

Fluorescence microscopic evaluation of multiplex stained urine cytospin slides was performed in a blinded fashion by 2 urologic pathologists (A. De Marzo, G. Netto) using an epifluorescence microscope with appropriate fluorescence filter sets (Nikon Eclipse 400; Nikon Instruments, Inc, Melville, NY). The slides were categorized for molecular cytology into 1 of 3 categories: (i) *malignant*, presence of cells displaying simultaneous markers of both prostatic origin and PC; (ii) *suspicious*, presence of cells displaying one or more of the cancer-associated markers but lacking definitive markers of prostatic origin or cells with positive markers but of suboptimal quality, thus precluding definitive categorization as malignant; (iii) *negative*, lacking cells displaying markers of the malignant phenotype, although cells of prostate origin may be present. When both slides from each case were diagnosed as negative, the cases were considered negative for PC.

2.6. Evaluation of Papanicolaou-stained slides

After complete evaluations of the cytospin slides stained for immunofluorescence, the subset of slides from patients with prospective urine samples collected before biopsy or transurethral resection (35 cases) were processed for standard cytopathologic examination. This was accomplished by coverslip removal, washing to remove antifade mounting medium, and staining by the technique of

Papanicolaou (PAP) [17]. The PAP-stained slides were evaluated by a cytopathologist with expertise in urine cytopathology (S. Ali) and scored for the presence of glandular epithelial cells, which were characterized as benign, atypical/suspicious, or malignant per standard cytologic convention.

2.7. Statistical analysis

Differences between age and PSA between study subjects with or without PC were analyzed by the Mann-Whitney test. The immunofluorescence staining results were analyzed by the Fisher exact test.

3. Results

3.1. Validation of multiplex staining with LNCaP cells in urine

Fig. 1 shows representative staining of cytospin slides derived from LNCaP-spiked urine fluorescently stained with individual antibodies directed against (i) the prostate-specific marker Nkx3.1, (ii) AMACR, which is overexpressed in PC, and (iii) nucleolin, which serves to highlight abnormal nucleoli—a hallmark of PC cells. Combined staining for multiple markers was also achieved. For example, a combination of AMACR positivity, prominent and multiple nucleoli (nucleolin staining), plus nuclear Nkx3.1 positivity (Fig. 1D) allowed LNCaP PC cells to be readily distinguished from other cell types present in the sample. Rare normal cells were positive for single markers, typically staining weakly for either nucleolin or AMACR, but no normal cells stained positively for 2 or more of the biomarkers used.

When observed, autofluorescence was primarily cytoplasmic and exhibited sample-to-sample variability as well as variability between differing cell types. Although bright at times, it did not interfere with the detection of our marker panel because of the facts that 2 of the markers, nucleolin and Nkx3.1, are nuclear, whereas AMACR positivity manifests as a distinctive punctate cytoplasmic staining pattern restricted to the single color channel used for the fluorescent secondary detection reagent; in contrast, cellular autofluorescence tended to exhibit a more homogeneous pattern and was also present in more than one color channel.

Regarding cell recovery and reproducibility, by varying the numbers of LNCaP cells added into the urine, we found that we could reproducibly detect LNCaP cells in the cytospin slides 100% of the time from an input number of 1000 LNCaP cells; however, cells were only detected on the cytospin slides 50% of the time when 100 LNCaP cells were introduced, and no cells were detected when only 10 LNCaP cells were added to the urine samples.

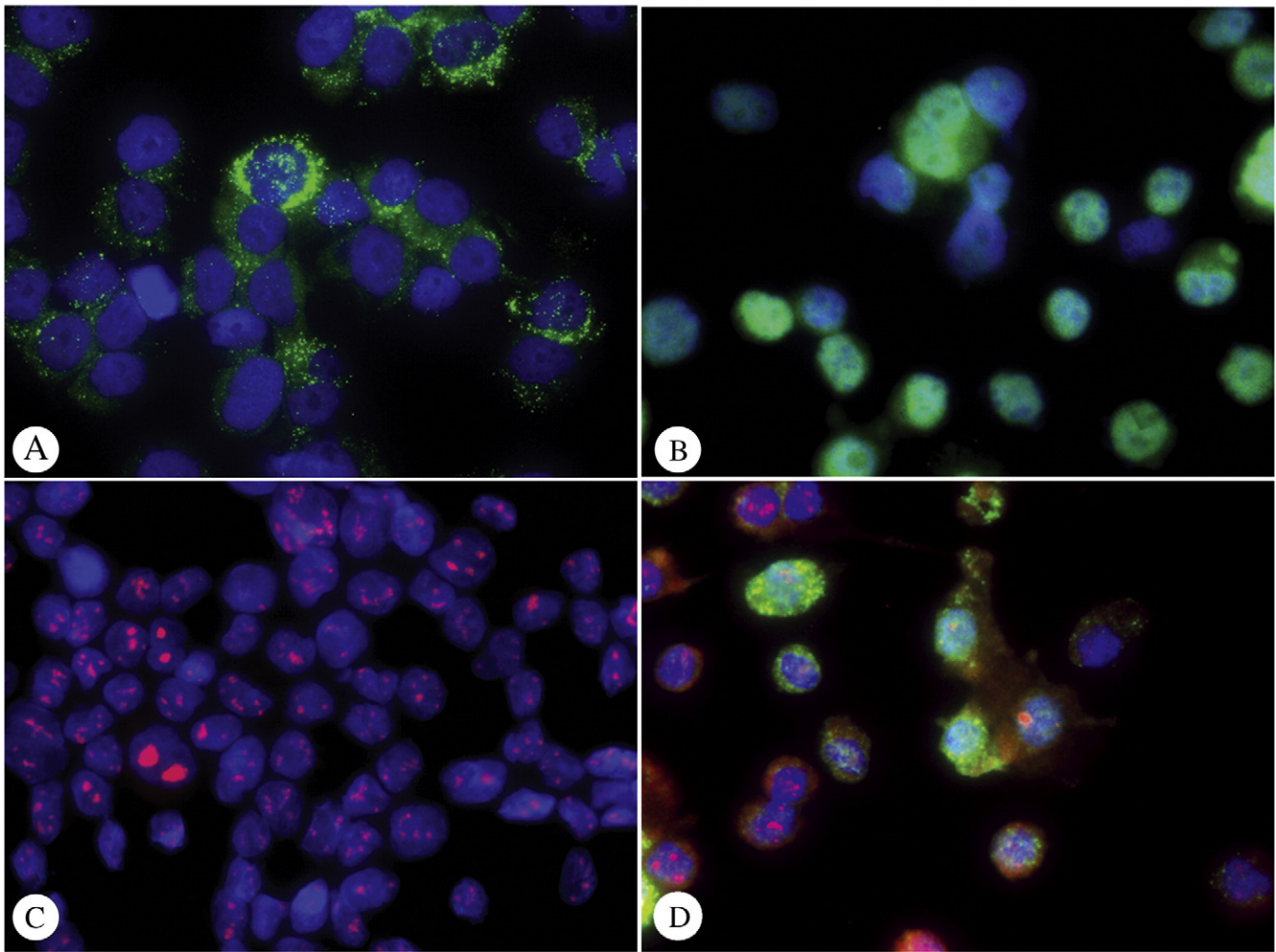


Fig. 1 Immunofluorescence staining of LNCaP PC cell cytopspins. LNCaP cytospin preps were stained with (A) anti-AMACR antibody detected in green (punctuate cytoplasmic staining pattern); (B) anti-Nkx3.1 (nuclear pattern) and (C) antinucleolin (nucleolar pattern); (D) combined fluorescent staining for AMACR, Nkx3.1, and nucleolin. In all cases, nuclei are costained blue with the DNA-binding dye DAPI (original magnification $\times 400$).

3.2. Staining of urine samples

On the cytospin slides made using clinical urine samples, the number of cellular elements (including all cell types, see below) present varied markedly from case to case. Of the 35 study samples, 19 cases displayed low to very low cell numbers (a few hundred cells or less), 8 cases displayed moderate cell numbers (a few thousand cells), and 6 cases displayed high cell numbers (several thousand cells). The presence of corpora amylacea was noted on 11 (31%) of the 35 cases, indicating that the prostate was contributing material to at least a subset of the urine sediments. Six of the 11 specimens with corpora also had moderate to high cellularity. In 2 cases (cases 12 and 31), the cytospin slides contained virtually no cells and, thus, were considered inadequate for evaluation by multiplex fluorescence analysis and were classified as uninformative.

In 27 of the 35 study cases, PC was detected on needle biopsy (Table 1). There were no significant differences in age or PSA between the cancer group and the negative biopsy group. Table 2 compares the molecular cytology results to the pathologic findings on needle biopsy. Among the 25 informative cases with biopsy-proven PC, 4 cases

Table 2 Performance of molecular cytology in clinical urine specimens			
Molecular cytology	Cancer status		NED voided urines (n = 15)
	Positive (n = 25)	Negative (n = 8)	
Positive	4	0	0
Suspicious	5	0	0
Negative	16	8	15
Uninformative	2		

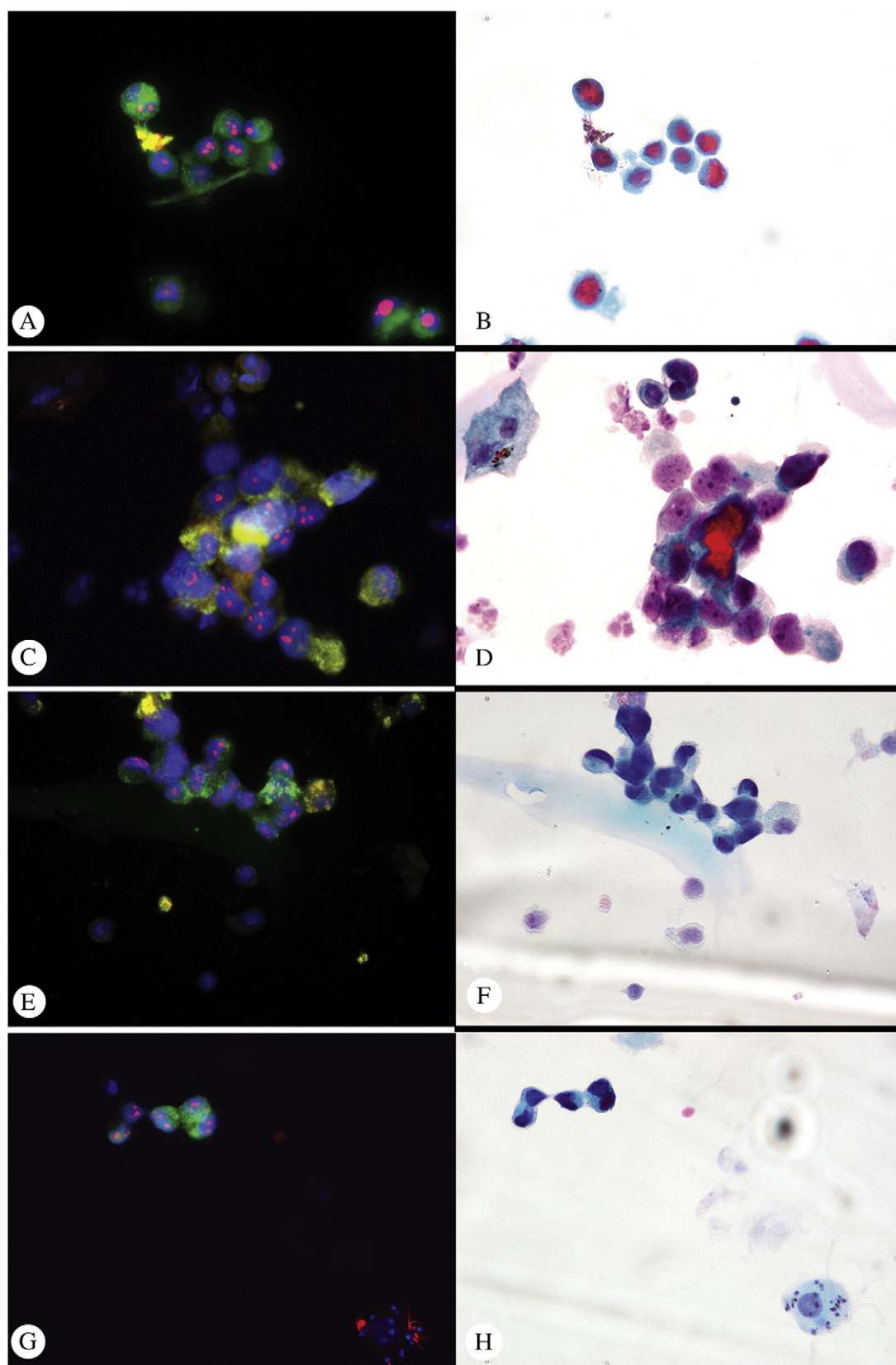


Fig. 2 Multiplex staining of urine sediments obtained from clinical urine specimens. A, C, E, and G, Combined fluorescent staining for AMACR (green, cytoplasmic), Nkx3.1 (green, nuclear), and nucleolin (red, nucleolar) strongly suggests the presence of exfoliated PC cells in these urine specimens. B, D, F, and H, Same specimens stained by the PAP procedure.

were classified as malignant by molecular cytology, 5 cases were considered suspicious, and 16 cases were considered negative. Positive cases typically contained small clumps of suspicious or malignant cells, although solitary positive cells were sometimes observed (Fig. 2). Suspicious cases generally had cells with very weak or absent Nkx3.1 staining, although they displayed staining for AMACR and multiple or large nucleoli. The group of 15 control patients and patients with NED were all negative by molecular cytology.

By combining the 2 abnormal categories (suspicious and malignant), the overall sensitivity of multiplex staining to detect PC in urine specimens after prostatic manipulation was 33% (9/27), with a specificity of 100% (8/8). Notably, of the 9 cases judged to be abnormal, 7 were from slides having cellularity in the moderate-high range. No significant differences were found between the 3 molecular cytology categories and any of the clinicopathologic variables in this cohort.

The study cohort consisted of 27 men with PC and 8 without. Pathologically, 6 of the PC cases had Gleason pattern 4 disease, whereas the other 21 had only Gleason pattern 3 disease. Four (67%) of the 6 cases with pattern 4 produced molecular cytology results that were either suspicious or positive on multiplex immunofluorescence cytology. In one of the other cases, the specimen was deemed inadequate in terms of cellularity (likely due to improper collection), and in the other, a pattern 4 + 3 case was missed (negative urine sediment). Although the yield of molecular cytology for cases of high-grade PC (Gleason pattern 4) was much higher than for the pattern 3 cases, the subset under analysis was smaller.

3.3. Comparison of molecular cytology and standard cytopathology

We further wished to determine how these same slides would be judged by standard cytopathology. In an attempt to address this question, we carefully removed the coverslips and submitted the slides for PAP staining and blinded cytopathology review. A range of cell types, typical of those seen in urinary cytology specimens, were noted on the PAP-stained slides, including urothelial cells, umbrella cells, squamous cells, inflammatory cells, renal tubule cells, and mature sperm. Of the 27 biopsy-confirmed cancer cases, 3 were diagnosed as having atypical or suspicious glandular epithelial cells and one case was diagnosed as containing frankly malignant epithelial cells consistent with prostate adenocarcinoma, giving a sensitivity of 15% (4/27) for PC detection by standard cytology when suspicious and malignant categories were combined. The single case deemed malignant by standard cytology had also been deemed malignant by molecular cytology, and upon review, this assessment turned out to be based on the same group of abnormal cells (Fig. 2C and D). All 4 cases deemed

atypical/suspicious or malignant by PAP staining were from cases that had moderate to high cellularity on the cytospin slides. Of the 2 cases considered uninformative by multiplex fluorescence staining because of low cellularity, one (case 12) was likewise deemed uninformative by standard cytology, whereas the other (case 31), although scant, was nonetheless characterized as normal. As with molecular cytology, all cases negative for cancer on biopsy were deemed benign by cytopathology.

After the blinded cytopathology review, we reexamined the slides from the discordant cases that had first been deemed abnormal by molecular cytology but later diagnosed as benign by standard cytopathology. In 5 of these cases, we were able to find the identical cells on the PAP-stained slides that had previously been judged abnormal on the molecular cytology slides, and these specific cells were presented to the cytopathologist for review. In one case, the cells had been classified previously as benign-appearing epithelial glandular cells, likely of prostatic origin. In the remaining 4 cases, the cells had not been noted during the initial review of the PAP-stained slides. In 3 of these cases, the cells were described, on second review, as small cells with high N/C ratios with poorly preserved smudgy chromatin without visible or prominent nucleoli (Fig. 2G and H). It was further noted that although the small cell sizes and high N/C ratios would be consistent with prostate adenocarcinoma, the morphological preservation was suboptimal and precluded such a diagnosis. In the remaining case, the cells were described as a 3-dimensional glandular fragment with smudgy chromatin and difficult to discern nucleoli.

4. Discussion

More than 600 000 diagnostic prostate biopsy procedures are performed annually in the United States. Prostate biopsies are not without risk and inherently undersample the prostate. The false-negative rate of initial biopsy has been estimated as high as 34%. Indeed, some 20% to 35% of patients sent for repeat biopsy are diagnosed with cancer not detected on initial biopsy [18,19]. On the other hand, many men lacking cancer on first biopsy do not have clinically detectable PC (true negatives) despite the continued presence of suspicious DRE or PSA results. Thus, patients whose initial biopsy results are negative present a serious clinical dilemma because of our inability to differentiate those patients at high risk for harboring undetected cancer who might benefit from immediate repeat biopsy from those unlikely to have cancer who could be spared unnecessary additional biopsies and their associated morbidity and cost. Men in this situation understandably experience much anxiety because of these uncertainties, and many will ultimately undergo multiple repeat biopsy procedures. In this regard, a noninvasive test to help augment current screening and testing modalities would be of enormous benefit.

After the successful demonstration by Papanicolaou in 1942 of his cytologic staining method for detecting exfoliated malignant cells of the uterine cervix, clinical researchers eagerly sought to apply the technique to detect cancer in bodily fluids [17]. With respect to the male genitourinary (GU) tract, although successful in detecting bladder cancer cells in urine sediments, the method failed to yield similar results for other types of GU cancer, including PC. Despite consistently high specificities, attempts at detecting PC cells by urine cytology were plagued by low sensitivities because of the relatively low numbers of PC cells present in urine cytology preps, plus difficulty in differentiating malignant prostate cells from the other cells and debris present [3-6,20,21]. Despite the inability of routine cytology to reliably detect PC cells in the urine, it was clear that, at least in a subset of cases, intact exfoliated PC cells could sometimes be recovered from urine samples or EPF.

The recent application of molecular techniques to the study of PC has led to the identification of several novel molecular alterations present in PC cells (eg, increased expression of the enzyme AMACR, abnormally short telomeres, and recurrent chromosomal translocations involving members of the ETS family of transcription factors, to name just a few) [13,22-26]. With the abandonment of the cytologic approach, current efforts are now focused on detecting such molecular changes in the urine or EPF [7-12,15].

Paralleling the advances in biomarker discovery, significant advances in antibody and in situ nucleic acid detection methods have also been made, such as tyramide signal amplification, rolling circle amplification, and the advent of fluorescent quantum dot secondary detection reagents [14,27,28]. Considering the expanding pool of candidate PC biomarkers, plus these modern tools for sensitive marker visualization that were unavailable to previous researchers attempting urine cytology, we felt that revisiting a cell-based approach using molecular cytopathology to detect PC cells in urine samples could be rewarding [29]. Specifically, we hypothesized that PC cells isolated from the urine could be stained simultaneously for a panel of molecular markers and imaged using fluorescence microscopy, thus providing a sensitive, noninvasive, and unambiguous method for their detection. Such a method would include (i) a clear delineation of prostate cells from other cell types present in urine samples, such as lymphocytes and cells originating from other portions of the GU tract (eg, bladder, urethra, or kidney), and (ii) identification of the subset of prostate epithelial cells that are malignant.

In this study, we have demonstrated the feasibility of using molecular markers to detect apparently intact PC cells in urine sediments. We first demonstrated the staining of LNCaP in urine with our marker set and then analyzed clinical urine samples in a cohort of men enriched with PC cases, some men who were biopsy negative, and some normal controls. For this pilot study, we specifically attempted to exclude men with conditions that might

confound our urine sediment assay: men with high-grade prostate intraepithelial neoplasia or urothelial carcinoma of the bladder, which could show positive AMACR or prominent nucleolin staining, respectively. Men with these conditions, as well as patients undergoing radical cystectomy for urothelial carcinoma without coexistent prostate adenocarcinoma, would be interesting subsets to study cytologically in the future with our multiplex technique for more accurate sensitivity and specificity determinations. Certainly, more PC-specific markers will be developed over the coming years; it will be of interest to optimize and study the most specific of these in such cohorts as well as in larger groups of men being screened and subsequently undertaking biopsy.

Several groups have demonstrated the ability to detect PC-specific biomarkers (eg, methylated DNA, protein, and RNA) in urine samples after manipulation of the prostate [7-12]. We, therefore, collected our urine specimens after transrectal prostatic massage, or “attentive DRE,” from men scheduled to undergo needle biopsy for suspicion of cancer. We then optimized methods for cell preservation, isolation, cytospin slide preparation, and multiplex staining for a panel of PC biomarkers. The specific markers and fluorescent secondary reagents used for their detection were chosen based upon consideration of the following: (1) cell type specificity, (2) species of origin of the primary antibody (restricts which secondary may be used), and (3) specific subcellular localization and staining character of the marker. These considerations not only presented some restrictions on which markers could be used together without interfering with one another, but also allowed for economy in the numbers of colored fluorescent secondaries used. For example, the anti-Nkx3.1 antibody homogeneously labels the nuclei of prostate epithelial cells, whereas the anti-racemase antibody yields strong punctate cytoplasmic staining in PC cells. Given their restriction to different spatially distinct cellular compartments (nucleus versus cytoplasm), the same colored secondary antibody (green in this example) can be used for the detection of both of these rabbit-derived primary antibodies without interference. There is no question that more accurate and more specific markers will be developed over the coming years—ours represents an attempt at developing a diagnostic panel given contemporary data and available reagents.

By assaying various combinations of antibodies and staining conditions, we decided upon a set of 3 antibodies (anti-Nkx3.1, anti-nucleolin, and anti-AMACR) for use in evaluating the feasibility of detecting PC cells by simultaneous fluorescent staining of cytospins from clinical urine specimens. Table 3 contrasts the expected staining phenotype of PC cells versus normal cells using this particular combination of markers. Elevated staining of AMACR, prominent nucleoli, and nuclear morphological abnormalities revealed by DAPI staining were used as markers of PC cells, whereas Nkx3.1 positivity confirmed their prostatic epithelial origin [15,22,25,30-32]. Other potentially useful markers of prostate epithelial cells such as prostate-specific

Table 3 Expected staining characteristics of PC versus normal cells in urine sediments for combined immunofluorescence

Marker	Subcellular localization	Cell staining phenotype: normal	Cell staining phenotype: PC
Anti-AMACR	Cytoplasm	Negative weak	Strong punctuate pattern
Anti-nucleolin	Nucleolus	Absent or weak, single nucleolus	Strong, prominent, and/or multiple nucleoli
Anti-Nkx3.1	Nucleus	Homogeneous nuclear stain in normal prostate epithelial cells, other cell types negative	Homogeneous nuclear stain

membrane antigen, PSA, and telomere fluorescence in situ hybridization were also assayed but did not perform well in combination with the other antibodies or lacked specificity in our assays (data not shown).

Regarding assay performance, there were no false positives, and thus, specificity was 100%. Sensitivity for PC detection was 36% by molecular cytology, which, although somewhat low, provides a proof of principle for this new approach. A recent multicenter study with multiplexed quantitative methylation-specific polymerase chain reaction assay demonstrated 53% to 55% sensitivity and 76% to 80% specificity for PC detection with urine obtained similarly (after DRE) [33]. Four of (67%) 6 cases with a higher grade (Gleason pattern 4) were deemed suspicious or positive by our assay, with one of the other 6 specimens having been judged acellular and thus inadequate from a collection standpoint. We hypothesize that in the rare instances of a virtually acellular sediment, patients either provided a midstream rather than an initial urine sample after attentive DRE, or the DRE was not vigorous enough. Alternately, in some cases, prostatic material may not be shed in sufficient quantity to be detectable in initial urine samples (at least by our current assays). Indeed, the main problem we encountered with the clinical specimens was low cellularity. Only 37% of samples yielded moderate to high cellularity on the cytospin slides. The fact that 78% of the samples displaying suspicious or malignant cells were cases with moderate to high cellularity supports the importance of obtaining adequate numbers of cells. This is likely to be especially important in cases where cancer cell numbers are limited, a situation reasonably to be expected with the small volume cancers currently typically detected in the PSA era. Currently, we are pursuing methods for stabilizing the pH and osmotic balance of the urine specimens in an attempt to help overcome this problem [34]. More thorough prostatic massage techniques, faster sample processing, accurate instruction to patients on providing initial post-DRE samples, and alternate fixation strategies may improve cell yields. In addition, alternative cell isolation strategies, such as the use of immunomagnetic beads (eg, Dynabead Epithelial Enrich or CELLextion beads; Invitrogen, Carlsbad, CA) or filtration in combination with automated fluorescence microscopy may also lead to improvements [35].

In summary, we have provided a proof of principle for the direct detection of PC cells from urine samples collected in the clinic. The assay was able to detect very small

numbers of cancer cells and was 100% specific. Although the sensitivity of the molecular cytology assay was relatively low, it did outperform standard cytology on the same samples, and it is reasonable to expect an increase in sensitivity with further refinement of the assay and development of better molecular targets. Molecular cytology, perhaps coupled with assays for soluble cancer biomarkers in the urine currently under development, should prove useful in solving the clinical dilemma of how best to proceed with men who are suspected of harboring PC but whose initial needle biopsy result is negative. In such patients, the finding of PC cells in postbiopsy urine would strongly imply undetected cancer and indicate the need for immediate rebiopsy. In addition to addressing this significant clinical problem, our assay could conceivably prove useful in other ways, such as for primary cancer detection after DRE, for monitoring disease status in PC patients who opt for expectant management (eg, “watchful waiting” or “active surveillance”), or for monitoring disease status in men who have undergone non-extirpative treatment approaches such as cryotherapy or radiotherapy. An assay such as this might also be useful to diagnose PC in patients who are unsuitable for biopsy because of hemorrhagic diathesis, need for constant anticoagulation, or severe medical comorbidities. Furthermore, it is conceivable that some qualitative or quantitative measure of PC cells found in the urine (eg, absolute numbers of cancer cells present) may correlate with tumor aggressiveness and thus also be of use in prognostication (such as Gleason grade), though this remains highly speculative. We believe the current study provides a compelling rationale for the continued development of methods for detecting PC in the urine by the same standard that is currently used for definitive diagnosis by pathologists in needle biopsies—direct visualization of the cancer cells themselves.

Acknowledgment

The authors express their gratitude to Karen Plowden of the Department of Pathology, Johns Hopkins Medical Institutions, Baltimore, MD, for her assistance in the use of the Surepath centrifugal technique for the isolation of cells from urine specimens. They also thank Tsuyoshi Iwata of the Department of Pathology, Johns Hopkins Medical Institutions, for technical advices.

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Endoglin (CD105) as a urinary and serum marker of prostate cancer

Kazutoshi Fujita, Charles M. Ewing, David Y.S. Chan, Leslie A. Mangold, Alan W. Partin, William B. Isaacs and Christian P. Pavlovich*

Brady Urological Institute, Johns Hopkins Medical Institutions, Baltimore, MD

We have previously shown that endoglin (CD105) is upregulated in prostatic fluid of men with large volume prostate cancer. We chose to assess endoglin levels in urine and serum from men with prostate cancer or at increased risk for the disease: Urine samples were collected after digital rectal examination (DRE) from 99 men whose cancer status was confirmed by biopsy, and serum samples were collected from 20 men without prostate cancer at low risk for the disease and from 69 men diagnosed with prostate cancer that subsequently underwent radical prostatectomy (30 pT2, 39 pT3). Endoglin levels were assessed by ELISA. Urinary endoglin was elevated in men with biopsy-positive prostate cancer compared to biopsy-negative men ($p = 0.0014$). Urinary endoglin levels in men with prostate cancer correlated with radical prostatectomy tumor volume. The area under the receiver operating characteristic (ROC) curve was 0.72 for urinary endoglin and 0.50 for serum prostate-specific antigen (PSA; sensitivity for cancer detection 73%, specificity 63%). There were no differences in serum endoglin between normal and cancer cases, but there were increases in serum endoglin in non-organ confined (NOC, pT3+) versus organ-confined (OC, pT2) cases ($p = 0.0004$). The area under the ROC curve was 0.75 for serum endoglin and 0.63 for PSA for predicting NOC status, with a sensitivity of 67% and a specificity of 80%. In conclusion, elevations in post-DRE urinary endoglin suggest there may be value in further studying endoglin as a urinary biomarker of prostate cancer. Endoglin levels in both urine and serum may aid in prostate cancer detection and prognostication.

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Key words: endoglin; CD105; prostate cancer; biological markers; clinical markers

Prostate cancer is known to be clinically heterogeneous, with some cases presenting in an indolent fashion and others widely metastatic at diagnosis. Prostate-specific antigen (PSA), digital rectal examination (DRE) and biopsy Gleason score are the 3 clinical tools typically used to stratify newly diagnosed men into low-, intermediate- or high-risk prognostic groups.¹ No other marker in routine use significantly adds to either the diagnostic or prognostic power of these clinical parameters. Nevertheless, there is a need for additional markers of early or aggressive/advanced prostate cancer, and the search for these is ongoing and increasingly technology-driven.^{2–4}

We have previously used a human cytokine array to identify cytokines in expressed prostatic fluid associated with large volume prostate cancers. We found that a variety of growth factors, cytokines and markers of angiogenesis were upregulated in prostatic fluid from such cases.⁵ One of the 20 most upregulated molecules (see ref. 5 Supplementary Table) was endoglin (CD105), a type I homodimeric integral transmembrane glycoprotein and accessory TGF- β receptor; another was the endoglin ligand activin-A.⁶ Given these common pathway findings, we selected endoglin for further study.

Endoglin is primarily expressed in proliferating vascular endothelial and smooth muscle cells, and is highly expressed on endothelial cells during tumor angiogenesis and inflammation. It has weak or negative expression in normal tissues. Endoglin is expressed in prostate microvasculature in association with prostate cancer, and is increased in the serum of patients with colorectal, breast and lung cancer metastases.^{7,8} Immunohistochemical analysis has shown endoglin to be expressed not only by endothelium associated with prostate cancer, but also by some prostatic intraepithelial neoplasia (PIN) and prostate cancer epithelial cells and

associated stromal components.⁹ Recently, soluble endoglin has been shown to be of independent prognostic value as a serum indicator of prostate cancer metastasis to pelvic lymph nodes and of biochemical recurrence after prostatectomy.^{10,11} Whether endoglin may serve as a marker for prostate cancer in locally-derived tissue (biopsies), or biofluids (expressed prostatic secretions, post-DRE urine) has been little studied.

We set out to assess whether endoglin levels could predict the presence of prostate cancer and/or correlate with advanced disease. Because endoglin is a local marker of vascular proliferation in response to injury and/or angiogenic stimulation, we felt that assessing endoglin levels from the prostatic microenvironment more directly might have merit: To this effect we assayed urine samples collected following DRE which is known to be enriched with prostatic secretions, from patients with and without prostate cancer. In addition, we assessed endoglin in archival serum samples from men with and without prostate cancer to assess its potential as a cancer biomarker.

Material and methods

Sample collection

Urine samples were collected in the Urology Clinic. Approval was obtained from our Institutional Review Board before initiating the study and all patients provided written informed consent prior to providing urine samples. Initial voided urine specimens (10–100 ml) were prospectively collected from 99 men with known prostate cancer or with an indication for prostate biopsy, immediately following DRE during a single office visit. In all cases, the DRE was an approximately 30-sec examination involving 3 finger strokes per prostate lobe and was done either 6 weeks or more after diagnostic biopsy, or just prior to diagnostic biopsy. In some men a urine sample was also collected prior to DRE. Voided urine specimens were kept at 4°C for up to 4 hr prior to centrifugation for 10 min at 1,000g to remove sediments and then urine supernatants were kept at –80°C until analysis. In addition, 89 archival serum samples were obtained from our biorepository and linked to information about patient prostate health status and other relevant demographic and pathologic data.

Enzyme-linked immunosorbent assay

Endoglin levels were measured by enzyme-linked immunosorbent assay (ELISA). A human Duo set (R&D Systems, Minneapolis)

Abbreviations: DRE, digital rectal examination; ELISA, enzyme-linked immunosorbent assay; NOC, non-organ confined; OC, organ-confined; PIN, prostatic intraepithelial neoplasia; PSA, prostate-specific antigen; ROC, receiver operating characteristic; TGF, transforming growth factor; TP, total protein.

Grant sponsor: National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK); Grant number: 1K23DK071262; Grant sponsor: Department of Defense (DOD); Grant number: W81XWH-05-1-0167; Grant sponsor: National Cancer Institute (NCI); Grant numbers: 5 P50 CA58236, 5U01 CA86323-08.

*Correspondence to: Brady Urological Institute, A-345, Johns Hopkins Bayview Medical Center, 4940 Eastern Avenue, Baltimore, MD 21224, USA. Fax: +410-550-4188 or +410-550-3341.

E-mail: cpavlov2@jhmi.edu

Received 8 July 2008; Accepted after revision 28 August 2008

DOI 10.1002/ijc.24007

Published online 19 September 2008 in Wiley InterScience (www.interscience.wiley.com).

lis, MN)) was used to detect endoglin in urine and serum. Briefly, 96-well microplates were coated with capture antibody and incubated overnight. After the blocking with 10% BSA in PBS for urine and 25% FBS in PBS for serum, samples were added (100 µl/well) in duplicate for incubation for 2 hr at room temperature. Detection antibodies were subsequently added (100 µl/well) and incubated for 2 hr at room temperature. Incubation with streptavidin-horseradish-peroxidase (for 20 min) was followed by detection with 3,3',5,5'-tetramethylbenzidine (TMB) for 20 min. The reaction was stopped by the addition of 1.5 M H₂SO₄. Plates were read at 450 nm wavelength on a microplate reader (PHERA star, BMG Labtech, Durham, NC). All reactions were done at room temperature. Serum samples were assayed at a 4-fold dilution.

ELISA data from urine samples were normalized by total urinary protein or urinary creatinine levels as measured by Dade Dimension RxL. Serum ELISA data were not normalized.

These data were analyzed by cancer grade on biopsy (Gleason score 6 vs. ≥7) and, for the 36 radical prostatectomy cases, by pathologic stage, pathologic grade (Gleason score 6 vs. ≥7) and tumor volume (minimal-moderate vs. extensive). After assessing cancer diameters on all radical prostatectomy sections, specimens with minute foci of cancer with a maximum tumor area < 15 mm² were termed "minimal" disease, whereas specimens with a maximum tumor area >80 mm² were termed "extensive" disease; tumors of in-between sizes were termed "moderate" disease.⁵

Data analysis and statistics

Statistical analyses were done using GraphPad Prism 4.0 for Windows. Mann-Whitney tests were used to analyze the difference of 2 categories. Power calculations were performed based on serum endoglin levels reported in the literature to detect a 5 ng/ml difference between cancer and control patients (one standard deviation).^{8,10,11} With a two-sided alpha = 0.05, and given a limited number of patients in the control group (20) due to specimen availability, power was >0.80 to detect a difference between groups. Biochemical and clinical prostate cancer recurrence data were available for 39 patients with non-organ confined (NOC) disease. Kaplan-Meier recurrence curves were generated for cases with low (<median) and high (>median) serum endoglin levels, and Log-Rank tests were used to analyze the differences. Statistical significance was defined as a *p* value < 0.05.

Results

Endoglin levels in urine

ELISA was used to quantitate the levels of urinary endoglin in a 99-man cohort of men with known prostate cancer or at increased risk of prostate cancer. Of these 99 men, 67 had biopsy-positive prostate cancer (40 were biopsied 6 weeks or more prior to post-DRE urine collection and 27 were diagnosed after post-DRE urine collection) and 32 were biopsy-negative, for an overall rate of prostate cancer in this cohort of 67.7%. The men with and without biopsy-positive prostate cancer were well-matched by age, PSA and DRE findings (Table I). Preliminary experiments demonstrated that endoglin levels were significantly higher in matched urine samples collected post-DRE compared to pre-DRE (mean endoglin in voided urine: 27.1 ± 16 pg/ml, mean endoglin in post-DRE urine: 68.9 ± 39 pg/ml), therefore post-DRE urine samples were used for all subsequent urinary analyses.

Endoglin levels were significantly higher in the urine of men with prostate cancer than in those without prostate cancer (Fig. 1a). Endoglin levels were normalized both to total urinary protein (TP) (Fig. 1b) and to urinary creatinine (Fig. 1c), but remained significantly elevated in the cancer cases regardless of the method of normalization (though normalization to total urinary protein was most discriminating). To assess whether endoglin levels might confer prognostic information in patients diagnosed with prostate cancer, we stratified those who underwent radical prostatectomy (*n* = 34) by stage (organ-confined (OC, pT2), NOC

TABLE I—PATIENT CHARACTERISTICS RESPECTIVE TO ANALYZED URINE AND SERUM SAMPLES

All	Urine samples		Controls		Serum samples		
					CaP Patients		
	Negative biopsy	Positive biopsy			pT2	pT3+	
No. of pts	32	67	20	69	30	39	
Median age (range)	62 (40–81)	60 (45–84)	56 (46–66)	61 (47–69)	57.5 (48–66)	62 (47–69)	<i>p</i> = 0.01
Median PSA (ng/ml)	5.4 (0.6–11.5)	5.05 (1.7–20.5)	1.05 (0.3–1.9)	5.29 (0.9–27.8)	4.7 (2.9–13.4)	6.5 (0.9–27.8)	<i>p</i> = 0.07
Suspicious DRE (%)	18.7	19.4	0	24.3	13.3	32.5	
Gleason Score							
6	—	43 (64%)	—	27 (39%)	17 (57%)	10 (26%)	
7	—	21 (31%)	—	35 (51%)	13 (43%)	22 (56%)	
8	—	1 (2%)	—	5 (7%)	0	5 (13%)	
9	—	2 (3%)	—	2 (3%)	0	2 (5%)	

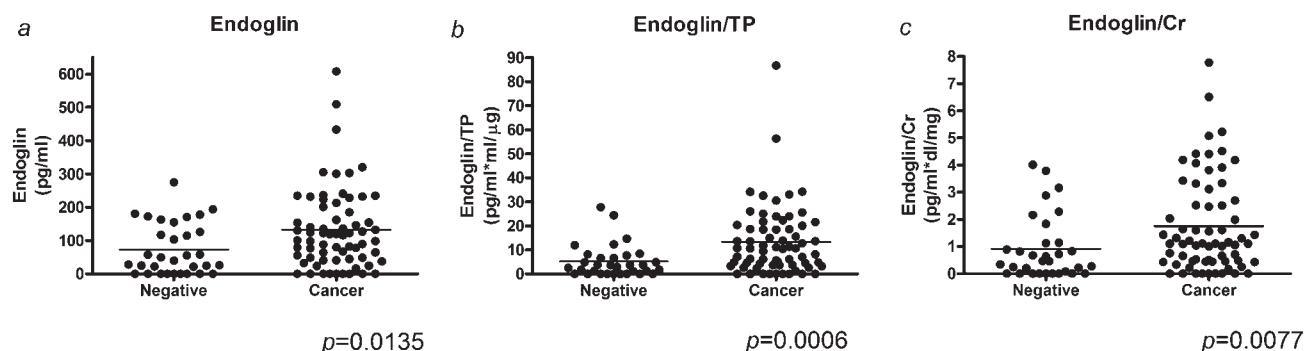


FIGURE 1 – Urinary endoglin collected after DRE in patients who had either a negative ($n = 32$) or positive ($n = 67$) biopsy for prostate cancer. (a) Urinary endoglin, (b) urinary endoglin/urinary total protein (TP), (c) urinary endoglin/urinary creatinine (Cr).

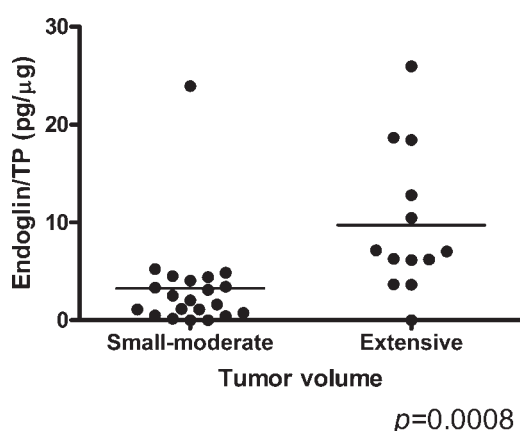


FIGURE 2 – Urinary endoglin/urinary total protein in patients with prostate cancer who subsequently underwent radical prostatectomy and had tumor volume assessed.

(pT3+), Gleason score (≤ 6 , ≥ 7) and tumor volume (minimal-moderate, extensive). Urinary endoglin levels were significantly higher in cases with high tumor volume (extensive prostate cancer, mean endoglin level = 9.73 ± 7.35 pg/ μ g, range: 0–25.95) compared to cases with smaller tumor volume (minimal/moderate prostate cancer, mean endoglin level = 3.25 ± 5.05 pg/ μ g, range: 0–23.4) ($p = 0.008$ (Fig. 2)). Mean urinary endoglin in men without prostate cancer was 73.2 ± 77.0 pg/ml (range: 0–274.8), and in those with prostate cancer was 132.4 ± 121.4 pg/ml (range: 0–608.3) ($p = 0.0135$). Mean endoglin levels normalized by TP of men without prostate cancer were 5.18 ± 6.8 pg/ μ g (range: 0–27.7), and those with prostate cancer were 13.4 ± 14.4 pg/ μ g (range: 0–86.7) ($p = 0.0006$). Mean endoglin levels normalized by urinary creatinine of men without prostate cancer were 0.92 pg/ml dl/mg ± 1.17 (range: 0–4.02), and those with prostate cancer were 1.75 pg/ml dl/mg ± 1.76 (range: 0–7.78) ($p = 0.0077$). There were neither significant differences in urinary endoglin levels by Gleason score or cancer stage, nor did men with benign biopsies but high urinary endoglin have an obvious increase in preneoplastic features such as PIN or atypia (data not shown). Urinary endoglin levels did not correlate with serum PSA or age.

The area under the ROC curve (AUC) for urinary endoglin was 0.72 (95% CI 0.61–0.82), in contrast to an AUC for PSA of 0.50 (95% CI 0.37–0.63) (AUC comparison $p < 0.01$) for cancer in our patient cohort (Fig. 3). The sensitivity and specificity at different endoglin/urinary TP cutoffs are listed on Table II.

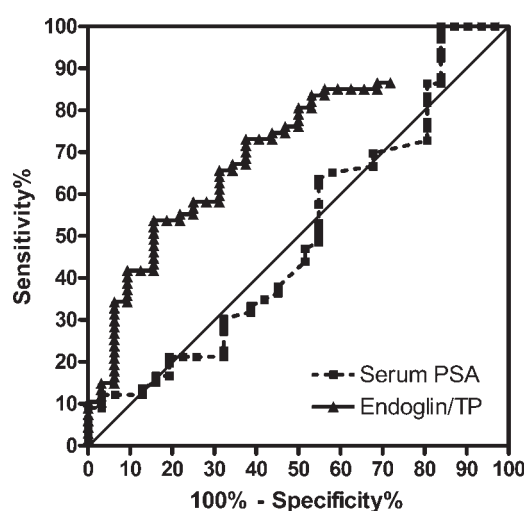


FIGURE 3 – ROC curves of urinary endoglin and serum PSA for the detection of cancer in our cohort.

TABLE II – URINARY ENDOGLIN NORMALIZED TO TOTAL URINARY PROTEIN (TP) AS A MARKER FOR PROSTATE CANCER IN MEN AT INCREASED RISK FOR PROSTATE CANCER (ABNORMAL PSA OR DRE)

Urinary endoglin/ TP cutoff	Sensitivity		Specificity	
	%	95% CI	%	95% CI
14.8	34.3	(23.1–46.9)	93.7	(79.1–99.2)
8.9	53.7	(41.1–66.0)	84.3	(67.2–94.7)
4	73.1	(60.9–83.2)	62.5	(43.6–78.9)
3.1	80.6	(69.1–89.2)	50	(31.8–68.1)
1.9	85	(74.2–92.6)	43.8	(26.3–62.3)

Endoglin levels in serum

Serum samples in a separate cohort of 89 patients with and without prostate cancer were also assessed for endoglin levels by ELISA (Table I). There was no overall difference in serum endoglin levels in men with prostate cancer compared to men without prostate cancer (16.9 ± 2.6 ng/ml, range: 9.4–25.5 vs. 18.1 ± 2.6 ng/ml, range: 13.8–21.6, respectively) (Fig. 4). However, among the 69 men with prostate cancer, endoglin levels were significantly higher in NOC (pT3+) (mean: 18.0 ± 3.6 ng/ml, range: 9.4–25.5) versus OC (pT2) disease (mean: 15.4 ± 2.3 ng/ml, range: 11.5–20.2) ($p < 0.01$). The men with prostate cancer were typically older, had higher PSA, and had more abnormal DRE findings than the men who did not have prostate cancer (Table I),

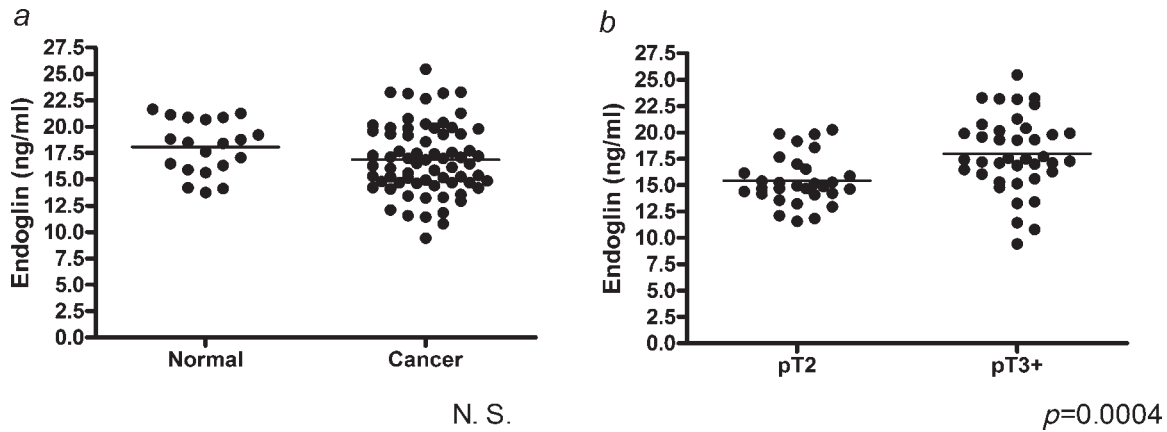


FIGURE 4 – Serum endoglin levels in patients (a) without prostate cancer (normal, $n = 20$) and with prostate cancer (cancer, $n = 69$), and (b) with OC prostate cancer (pT2, $n = 30$), and with NOC prostate cancer (pT3+, $n = 39$).

but in separate univariate analyses, no correlation was found between serum endoglin levels and age or Gleason score. The ROC curve for serum endoglin is compared to that for PSA to predict pT3+ disease (Fig. 5a), with an AUC for endoglin of 0.75 (95% CI 0.63–0.87) in contrast to an AUC for PSA of 0.63 (95% CI 0.50–0.77) (AUC comparison $p = 0.10$). The sensitivity was 67% and the specificity was 80% for the prediction of NOC disease with a serum endoglin cutoff of 17.0 ng/ml.

A subset of patients with NOC disease with (20) and without (19) postoperative PSA recurrence was compared by preoperative serum endoglin level, and no difference was found (18.6 vs. 17.3 ng/ml). Log Rank analysis for post-prostatectomy biochemical recurrence showed no significant difference between men in this subset with low versus high endoglin levels (<50%ile vs. >50%ile endoglin, $p = 0.21$) (Fig. 5b).

Discussion

We hypothesized that biomarkers associated with the development of prostate cancer and/or of its dedifferentiation can be measured from the prostatic microenvironment. Prostatic stroma and epithelium are known to be rich sources of cytokines and growth factors involved in the regulation of prostatic development, hypertrophy and neoplasia, as well as of inflammation and local immunity.¹² In previous experiments, we assayed prostatic fluid for cancer-associated proteins: in addition to increased amounts of cytokines such as HGF and IL18 binding protein-a, we noted increased CD105/endoglin and increased amounts of one of its ligands (activin-A) in expressed prostatic fluid collected from radical prostatectomy specimens with large volume cancers.⁵ In the present study, we show that endoglin is increased in urine collected after DRE from men with prostate cancer on biopsy compared to men without prostate cancer, and that post-DRE urinary endoglin levels are predictive of prostate cancer in a cohort of men at increased risk by PSA and DRE criteria (Fig. 3). This is the first assessment of the ability of endoglin to distinguish between benign and malignant prostate disease. In addition, endoglin levels measured from serum were predictive of NOC prostate cancer using an archival set of serum samples from men with and without prostate cancer.

Endoglin was assayed in the urine after DRE to directly (but minimally-invasively) assess its presence in the prostatic microenvironment *in vivo*. An attentive DRE exerts pressure on much of the prostate, and at least in theory allows for a sampling of secretions from the entire gland, unlike a prostate biopsy. It is known that initial voided urine obtained after DRE is enriched in prostatic proteins.¹³ We did not specifically assess whether the urinary endoglin we detected was a result of circulating and filtered endo-

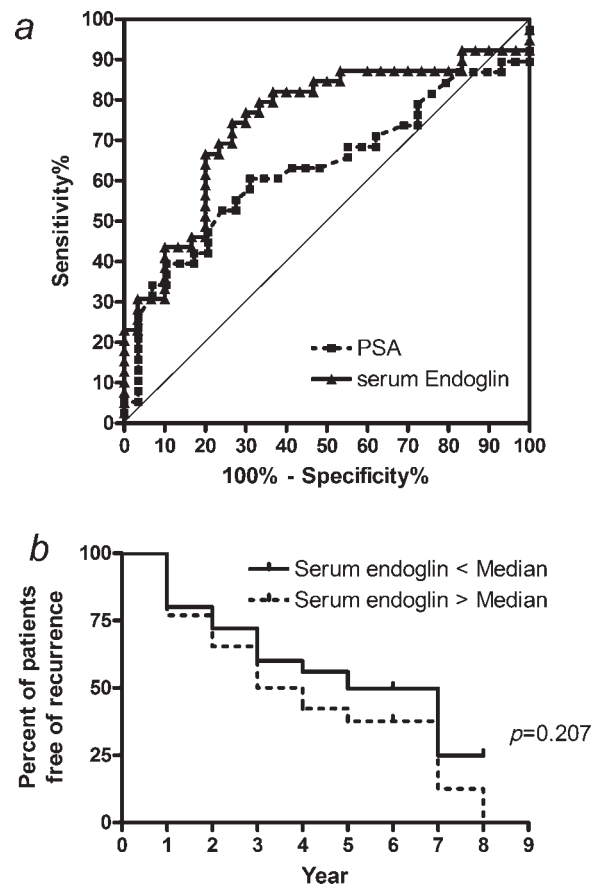


FIGURE 5 – (a) ROC curve of serum endoglin and serum PSA for the prediction of NOC disease in patients with prostate cancer on biopsy. (b) Kaplan-Meier recurrence curves for cases with low (<median) and high (>median) serum endoglin levels for 39 cases with documented NOC disease.

glin or a result of local prostatic endoglin. However, given that the assays were performed after prostatic manipulation, that we have previously found endoglin in expressed prostatic secretions, that only initial urine was collected as it coursed through the prostate after prostatic examination (“Voided bladder 3” samples, per Meares-Stamey),¹³ and that we normalized to total protein in the urine samples (which mostly comes from prostatic sources after a

DRE), we surmise that the endoglin we assayed was predominantly of prostatic origin. Urine is likely to become an increasingly powerful source of prostate-specific biomarkers,^{2,4} but until quantitative detection methods improve it may be reasonable to sample urine enriched in prostatic secretions rather than urine that is prostate secretion-poor (such as mid-stream urinalysis).

Serum PSA is an extremely powerful marker of prostatic disease, with tremendous diagnostic and prognostic utility, but it is not cancer-specific.¹⁴ Nevertheless, PSA and its isoforms are the sole prostatic serum markers in clinical use today, and PSA testing alone has changed the epidemiology of prostate cancer dramatically since its introduction in the 1980s.¹⁵ Our cohort of men who provided post-DRE urine samples had mean PSA levels between 5 and 5.5 ng/ml (*i.e.* elevated), and almost 20% had abnormal DRE findings (Table I). These men could be characterized as being at elevated risk for prostate cancer primarily based on PSA criteria. Our urinary endoglin test demonstrated better performance characteristics than PSA in this cohort of high-risk men; however, it is unclear how urinary endoglin would perform in a more generalizable male population, where PSA has significant clinical utility. Another caveat is that the cohort under study included men with known untreated prostate cancer as well as men at elevated risk for prostate cancer undergoing biopsy. Our overall cohort was thus weighted 2:1 with men harboring prostate cancer, in contrast to routine clinical settings in which biopsies yield a 1:2 prostate cancer detection rate. Therefore, although the performance of urinary endoglin in such a cohort was better than PSA, it remains to be tested in more routine clinical scenarios and on a larger scale.

We studied endoglin levels from archival serum samples in a separate cohort of men with and without prostate cancer. We did not find a significant difference in serum endoglin between men with and without prostate cancer, but a study with greater power might elucidate any difference that may have been missed. On the other hand, serum endoglin levels in men with pathologic stage III (NOC) disease were significantly greater than those in men with pathologic stage II (OC) disease. This statistically significant finding may not easily translate into a clinically useful pretreatment counseling tool because of the small differences in absolute levels.

Endoglin has recently gained attention in prostate cancer prognostication by work from the group from the University of Texas Southwestern that has had a longstanding interest in TGF- β related proteins and prostate cancer. They analyzed endoglin levels in archival serum from a large cohort of prostatectomy patients and showed an independent association between increased plasma endoglin and the presence of lymph node metastasis and biochemical recurrence after prostatectomy, suggesting this molecule may be a marker of and/or facilitate extraprostatic spread.^{10,11} Interestingly, our 2 groups have come upon endoglin in distinct manners, one from scientific analysis of TGF- β related pathways, and the other from cytokine profiling of prostatic fluid; consistently, both have demonstrated associations between endoglin and aggressive prostate cancer.

Because endoglin is a marker of pan-endothelial damage and angiogenesis, it is unlikely that circulating endoglin levels would be significantly affected by localized prostatic disease states—indeed, serum endoglin levels are affected by cardiovascular disease status, cholesteremia and cirrhosis.^{16,17} However, circulating endoglin is increased in metastatic disease states.^{8,10,11} Presumably, the angiogenic cascade necessary for metastasis is associated with systemic dysregulation of the TGF- β superfamily that results in an increase in detectable serum endoglin. Our finding of increased serum endoglin in NOC prostate cancer states is consistent with the notion of endoglin as a marker of advanced disease and supports the dramatic associations found between endoglin and metastatic disease by the U.T. Southwestern group. However, we were unable to show increased endoglin levels in patients with prostate cancer compared to patients without it. In addition, serum endoglin levels in our study patients differed from those in the other studies, which were somewhat higher even in localized disease states (20–40 ng/ml).^{10,11} Levels in our cohort ranged between 7.5 and 27.5 ng/ml (Fig. 4), while in the cardiovascular literature, levels in normal controls and in patients with familial atherosclerosis and/or in the setting of myocardial infarction average between 3 and 8 ng/ml.^{16,17} There is no standard assay for endoglin, but a variety of kits and antibodies are commercially available; it is possible that the specific ELISA used may be responsible for the range of levels reported in these different studies. Alternate explanations are that endoglin levels in serum and plasma may differ, and that endoglin levels may be affected by time of archival storage.

Endoglin's molecular role if any in prostate carcinogenesis and metastasis is unknown. Endoglin is known to be strongly upregulated in the endothelium of various tumors compared to normal tissues, suggesting that endoglin plays a significant role in tumor angiogenesis.¹⁸ Hypoxia transcriptionally induces endoglin expression *via* HIF-1, expression which is enhanced in the hypoxic setting by TGF- β .¹⁹ In turn, endoglin antagonizes the inhibitory effects of TGF- β 1 on human vascular endothelial cells; indeed normal cellular levels of endoglin/CD105 are required for the formation of new blood vessels.²⁰ Future work is required to determine the specific source of the endoglin detectable in the urine of prostate cancer patients, if it is bioactive, and what are its most important downstream targets with respect to prostate oncogenesis and prostate cancer progression.

Conclusions

Endoglin is an accessory TGF- β receptor transmembrane glycoprotein associated with angiogenesis and prostatic neoplasia that is present in prostatic fluid. Urinary levels of endoglin are increased in men with prostate cancer compared to levels in men without prostate cancer, and serum endoglin levels appear to correlate with increasing prostate cancer stage. Further studies are necessary to validate these initial observations.

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Monocyte Chemotactic Protein-1 (MCP-1/CCL2) Is Associated With Prostatic Growth Dysregulation and Benign Prostatic Hyperplasia

Kazutoshi Fujita,¹ Charles M. Ewing,¹ Robert H. Getzenberg,¹
J. Kellogg Parsons,^{2,3} William B. Isaacs,¹ and Christian P. Pavlovich^{1*}

¹*The Brady Urological Institute, Johns Hopkins Medical Institutions, Baltimore, Maryland*

²*Division of Urologic Oncology, Moores Comprehensive Cancer Center, University of California, San Diego, California*

³*Division of Urology, San Diego Veterans Affairs Medical Center, La Jolla, California*

BACKGROUND. Chronic inflammation is commonly observed in benign prostate hyperplasia (BPH), and prostate tissue often contains increased inflammatory infiltrates, including T cells and macrophages. Cytokines are not only key mediators of inflammation but may also play important roles in the initiation and progression of BPH.

METHODS. In order to determine what cytokines might be involved in prostatic enlargement, expressed prostatic secretions (EPS) from ex vivo prostates were analyzed by human cytokine antibody microarray and ELISA. Prostate epithelial cells (PrEC) and prostate stromal cells (PrSC) were used for ELISA, proliferation, and Western blot assays.

RESULTS. Monocyte chemotactic protein-1 (MCP-1/CCL2) was one of the most elevated proteins in secretions from large prostate glands. PrSC were found to secrete MCP-1; Western blotting showed that both PrSC and PrEC express the MCP-1 receptor CCR2 which by RT-PCR was the CCR2b isoform. Proliferation assays showed that MCP-1 stimulates the proliferation of PrEC, but not PrSC, and that a specific MCP-1 antagonist (RS102895) suppressed this effect. Conditioned medium from PrSC stimulated the proliferation of PrEC as well, an effect completely inhibited by both RS102895 and a neutralizing anti-MCP-1 monoclonal antibody. The inflammatory cytokines interleukin (IL)-1 β , interferon- γ , and IL-2 enhanced the secretion of MCP-1 from PrEC and PrSC. In addition, MCP-1 levels in EPS correlated with mRNA levels of the macrophage marker CD68 in the same secretions.

CONCLUSIONS. The cytokine MCP-1, of apparent prostatic stromal cell origin, may play an important role in prostatic enlargement and BPH, and is a candidate biomarker for these pathologic processes. *Prostate* © 2009 Wiley-Liss, Inc.

KEY WORDS: BPH; MCP-1; CCL2; PrEC; PrSC

INTRODUCTION

Benign prostate hyperplasia (BPH) is a common disease affecting older men caused by unregulated prostatic stromal and epithelial growth resulting in prostate enlargement, bladder outlet obstruction, and lower urinary tract symptoms [1]. Despite intensive research over the last several decades, the molecular mechanisms underlying prostatic enlargement and symptomatic bladder outlet obstruction remain obscure. However, androgens, estrogens, stromal-epithelial interactions, growth factors, and modifiable risk factors including obesity and diabetes likely play

important roles in the etiology of this hyperplastic process [2,3].

BPH is characterized histologically by an increased number of epithelial and stromal cells in the periurethral

Grant sponsor: NIH/NIDDK; Grant number: 1K23DK071262; Grant sponsor: Department of Defense; Grant number: W81XWH-05-0167.

*Correspondence to: Christian P. Pavlovich, The Brady Urological Institute, Johns Hopkins Bayview Medical Center, A345 4940 Eastern Ave., Baltimore, MD 21218. E-mail: cpavlov2@jhmi.edu

Received 19 August 2009; Accepted 24 September 2009

DOI 10.1002/pros.21081

Published online in Wiley InterScience

(www.interscience.wiley.com).

area of the prostate [4]. In addition, BPH tissues often have infiltrating lymphocytes and macrophages around the glandular elements, and chronic inflammation has been proposed in the pathogenesis of BPH [5–8]. These infiltrating cells produce cytokines, such as interleukin-6 (IL-6) and IL-8, which stimulate epithelial and stromal proliferation. Growth factors from stromal cells, such as epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) also stimulate epithelial cell proliferation.

We used a human cytokine array to search for cytokines in expressed prostatic secretions (EPS) that may be associated with prostate enlargement. In comparison to cytokines found in small volume prostates, a variety of growth factors and cytokines were found to be elevated in EPS from large prostates. One of these was monocyte/macrophage chemoattractant protein-1 (MCP-1/CCL2). MCP-1, or CCL2, is a member of the CC chemokine superfamily that plays a critical role in the recruitment and activation of monocytes during inflammation [9]. MCP-1 has been associated with BPH and chronic pelvic pain syndrome (CPPS) [10,11] and has also been shown to stimulate prostate cancer growth, invasion, and metastasis [12–14].

In this study, we sought to assess the association of MCP-1 levels in EPS with prostate size and further characterize MCP-1 and BPH in vitro using benign prostatic epithelial and stromal cell lines and MCP-1 inhibitors.

MATERIALS AND METHODS

Sample Collection

All samples were collected using IRB-approved protocols. EPS were collected by manually squeezing fresh ex vivo prostate glands immediately following radical prostatectomy for clinically localized prostate cancer, and gathering secretions obtained from the protruding apical urethral stump. The radical prostatectomy specimens were then submitted for routine weighing, formalin fixation, sectioning, and pathologic analysis as per standard protocol [15]. Secretions from patients subsequently determined to have minimal prostate cancer (defined as specimens with minute foci of a maximum tumor area of less than 15 mm² with Gleason sum ≤ 6) were chosen for this study. EPS were stored at -80°C until analysis.

Cytokine Antibody Array

A RaybioTM Human Cytokine Array Kit (Raybiotech, Norcross, GA) including 174 cytokines was used as described elsewhere [16]. Membranes immobilized

with capture antibodies were incubated with EPS samples [1 ml, in 10-fold dilution with Tris-buffered saline (TBS) and complete protease inhibitor cocktail tablets (Roche Diagnostics, Indianapolis, IN) for 2 hr at room temperature]. After extensive washing with TBS/0.1% Tween-20 (three times, 5 min each) and TBS (twice, 5 min each), membranes were incubated with biotin-conjugated anticytokine antibodies. Membranes were washed and then incubated with horseradish peroxidase-conjugated streptavidin (2.5 pg/ml) for 1 hr at room temperature. Finally, the signals were detected by the enhanced chemiluminescence system, followed by additional washing. Spots were visualized using enhanced chemiluminescence (ECL plus Western blotting system, Amersham Biosciences, Pittsburgh, PA). Membranes were exposed to Kodak X-Omat radiographic film for 1 min per image. Each film was scanned into TIFF image files, and spots were digitized into densities with Gel-Pro-Analyzer (Media Cybernetics, Bethesda, MD). The densities were exported into Microsoft Excel, and the background intensity was subtracted prior to analysis.

Cell Culture

Human prostate epithelial cells (PrEC) and prostate stromal cells (PrSC) were obtained from Lonza (Walkersville, MD). PrEC and PrSC were maintained in prostate epithelial cell growth media (PrEGM, Lonza) and stromal cell growth media (SCGM, Lonza), respectively, at 37°C containing 5% CO₂. Peripheral blood mononuclear cells (PBMC) were isolated from 10 ml blood from a healthy volunteer by density-gradient centrifugation with Ficoll-Paque Plus (GE Healthcare, Uppsala, Sweden). PBMC were then seeded in a six-well plate and incubated in RPMI (1% L-glutamate, 1% nonessential amino acids, 1% sodium pyruvate, 1% penicillin/streptomycin, 10% heat-inactivated FCS, and 14.4 mol/L beta-mercaptoethanol) for 3 hr. Nonadherent cells were removed by washing several times with HBSS and adherent monocytes on the plate were subjected to further experiment.

Measurement of MCP-1 in Cell Culture Conditioned Media

1×10^5 of each cell type were seeded in 24-well plates and 24 hr later media were changed to media with or without 10, 100 ng/ml of IFN- γ (R&D Systems), 10, 100 ng/ml IL-1 β (R&D Systems), and 10, 100 U/ml IL-2 (Roche, Nutley, NJ). After 48 hr of incubation, supernatants were collected, centrifuged at 1,000g for 10 min to remove cells and subjected to enzyme-linked immunosorbent assay (ELISA).

Enzyme-Linked Immunosorbent Assay (ELISA)

MCP-1 levels in EPS and in cell culture supernatants were measured by a human CCL2 (MCP-1) ELISA Ready-SET-GO kit (eBioscience, San Diego, CA). MCP-1 measurements were performed as per the manufacturer's recommendations. EPS and cell culture supernatants from PrSC were assayed at 16- and 4-fold dilutions, respectively.

Reverse-Transcriptase PCR

Total RNA was extracted from PrEC, PrSC, monocyte/macrophage, or EPS with the RNeasy Mini Kit (Qiagen, Valencia, CA). Total RNA was treated with DNase I (Qiagen). First-strand cDNA was produced with random hexamers as per the manufacturer's recommendations (Omniscript RT kit/Qiagen). PCR amplification of *CCR2a* and *CCR2b* was done with HotStar Taq Plus Master Mix (Qiagen) for cDNA of PrEC, PrSC, and monocyte/macrophage. Primers used were as follows: *CCR2a*, 5'-GAGACTCTTGGGATGACTCAC-3' (forward) and 5'-ACAGCGATGGAGCGTAT-3' (reverse); *CCR2b* 5'-GAGACTCTTGGGATGACTCAC-3' (forward) and 5'-TTATAAACCAGCCGAGACTTC-3' (reverse); *GAPDH*, 5'-ACCAGGGCTGCTTTTAACTCT-3' (forward) and 5'-GATGACAAGCTTCCCGTTCT-3' (reverse). Amplification conditions were as follows: 15 min at 95°C (one cycle) and 45 sec at 94°C; 45 sec at the annealing temperature of 56°C; and 60 sec at 72°C (35 cycles) and 72°C for 5 min (one cycle).

Real-time PCR was done to quantify mRNA levels of CD68 in EPS. PCR amplification mixtures (25 µl) contained 12.5 µl of iQ SYBR Green supermix (Bio-Rad Laboratories, Hercules, CA), 2 µl of a mixture of 2.5 µM reverse and forward primers, 5.5 µl of nuclease-free water, and 5 µl of cDNA template. Quantitative RT-PCR measurements were performed on an iQ5 real-time PCR Detection system with iCycler IQ Software (Bio-Rad Laboratories). PCR cycles proceeded as follows: Taq activation (X min, 95°C), then 40 cycles of denaturation (X sec, 95°C), annealing (X sec, 60°C), and extension (X sec, 72°C). The melting-curve analysis showed the specificity of the amplifications. The relative mRNA levels were estimated by standard method using β -actin as the reference gene. Primers used were as follows: CD68, 5'-CTACATGGCGGTGAGTACAA-3' (forward) and 5'-ATGATGAGAGGCAGCAAGATGG-3' (reverse); β -actin 5'-XXXXX-3' (forward) and 5'-XXXXX-3' (reverse).

Western Blot analysis

After washing with ice-cold PBS, cells were harvested in RIPA buffer (Pierce, Rockford, IL) supple-

mented with Halt protease inhibitor cocktail (Pierce). Total cellular protein concentrations were determined by using a BCA protein assay reagent (Pierce). Twenty-five micrograms protein of lysates were subjected to SDS-PAGE under reducing conditions and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). Membranes were immunoblotted with rabbit polyclonal antihuman CCR2 antibodies (Abcam, Cambridge, MA) followed by horseradish peroxidase-conjugated secondary antibodies and developed with the Super Signal West Pico Substrate kit (Pierce).

Cell Proliferation Assays

6000 PrEC cells and 1000 PrSC cells were seeded on 96-well plates and 24 hr later media were changed to media containing 0, 1, 10, and 100 ng/ml MCP-1 (R&D Systems) in PrEGM or SCBM. 6000 PrEC cells were also seeded on 96-well plates and 24 hr later media were changed to the media containing 0, 2.5, 5.0, and 10 µM RS102895 in PrEGM with 10 ng/ml MCP-1. The cell proliferation reagent WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolol]-1,3-benzene disulfonate) was added to each well 24, 48, and 72 hr later, as specified by the supplier (Roche). After 2 hr of incubation, WST-1 absorbance at 450 nm (OD₄₅₀) and 610 nm (OD₆₁₀) was measured and OD₄₅₀ was subtracted by OD₆₁₀. PrSC cells were also cultured in PrEGM media for 2 days and the conditioned media (CM) were harvested in 48 hr. The CM with 0, 2.5, 5.0, and 10 µM RS102895 or PrEGM media were also added in 96-well plates 24 hr after seeding. The CM from PrSC were also incubated with or without mouse monoclonal anti-MCP1 antibody (MAB279, R&D Systems) for 2 hr at room temperature and then added in 96-well plates 24 hr after seeding. The WST-1 reagent was added to each well 78 hr later, and absorbance was measured at 450 nm after 2 hr of incubation. Monocytes attached on the plate were incubated in PrEGM and CM were harvested 48 hr later. The CM from monocytes were also added to 96-well plates of PrEC, and 72 hr later the WST-1 reagent was added and absorbance measured in 2 hr.

Data Analysis and Statistics

Results were expressed as mean \pm SD. Statistical analyses were done using GraphPad Prism 4.0 for Windows. Mann-Whitney tests and Student's *t*-tests were used to analyze the difference of two categories in clinical samples and in vitro experiments, respectively. Statistical significance was defined as a *P*-value < 0.05 .

RESULTS

Cytokine Profile and MCP-1 Levels of Expressed Prostatic Secretions

The array-derived intensity values of cytokines from 21 prostates were calculated. Those from large prostates (>60 g; $n=8$) were divided by those from small prostates (<40 g; $n=6$) to calculate the relative n -fold change in intensity value. The ranked cytokine profile of the relative n -fold change is listed in Table I. The 16 most elevated cytokines in large prostates were analyzed, and of these, MCP-1 and IL-1 β demonstrated correlations with increasing gland weight in 21 prostates (Spearman's correlation test, $r=0.434$, $P=0.049$ and $r=0.419$, $P=0.059$, respectively). To validate these findings, we measured MCP-1 and IL-1 β levels by ELISA in EPS from a new cohort of 46 patients also with minimal disease postprostatectomy. Spearman's correlation analysis showed that MCP-1 levels were associated with increasing gland weight ($r=0.369$, $P=0.013$; Fig. 1) but that IL-1 β levels were not ($r=-0.015$, $P=0.9203$) (data not shown).

Prostate Stromal Cells Secrete MCP-1 and Both Prostate Epithelial and Stromal Cells Express CCR2

To localize MCP-1 by potential cell of benign prostatic origin, we cultured human PrEC and PrSC. Far higher levels of MCP-1 were found by ELISA in the supernatant of PrSC ($2,150 \pm 615$ pg/ml) compared to that of PrEC (27.0 ± 40.7 pg/ml; Fig. 2A). Western blot analysis demonstrated expression of the MCP-1 receptor protein CCR2, in both PrEC and PrSC (Fig. 2B). CCR2 has two variants, CCR2a and CCR2b, and

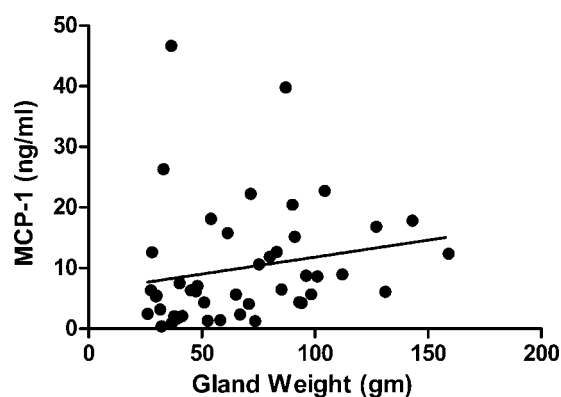


Fig. 1. MCP-1 levels in EPS ($n=46$) determined by ELISA compared to prostate specimen weight ($n=46$); Spearman's $r=0.369$, $P=0.013$.

RT-PCR analysis showed that PrEC and PrSC express primarily the CCR2b variant (Fig. 2C).

MCP-1 Stimulates the Proliferation of Epithelial Cells

To examine the effects of MCP-1 on benign epithelial and stromal cells, WST-1 proliferation assays were performed. MCP-1 significantly stimulated the proliferation of PrEC in 72 hr (10, 100 ng/ml MCP-1: $P<0.05$) but not of PrSC (Fig. 3A,B). The growth stimulation by MCP-1 of PrEC was blocked by RS102895, a specific antagonist of CCR2b ($P<0.05$; Fig. 3C).

To test the impact of MCP-1 secreted from stromal cells on epithelial cells, CM from PrSC were added to PrEC and proliferation was evaluated. CM from PrSC stimulated PrEC growth ($P<0.05$), and RS102895

TABLE I. Cytokine Profile of Prostatic Fluid

Cytokine	Ratio (L/S)	Average signal of L group (SD)	Average signal of S group (SD)
IL-1beta	5.08	4.75 (10.33)	0.94 (0.55)
IL-7	4.63	0.30 (0.37)	0.06 (0.10)
Activin A	4.47	2.75 (3.11)	0.62 (0.92)
MCP-1	3.67	103.47 (101.64)	28.2 (12.71)
IL-6	3.09	1.72 (3.56)	0.56 (0.53)
FGF-4	2.78	0.18 (0.21)	0.07 (0.10)
FGF-7	2.76	0.33 (0.53)	0.12 (0.29)
IGFBP-4	2.75	0.77 (0.97)	0.28 (0.36)
IGF-I	2.67	0.87 (1.10)	0.32 (0.31)
Endoglin	2.37	0.56 (0.68)	0.24 (0.58)
AgRP	1.98	3.16 (3.51)	2.25 (1.71)
BLC	1.96	7.10 (7.86)	3.63 (2.64)
I-309	1.95	1.87 (1.43)	0.96 (0.94)
PARC	1.85	9.11 (9.07)	4.93 (3.74)
FGF-6	1.77	0.43 (0.56)	0.24 (0.60)
GM-CSF	1.76	3.80 (4.56)	1.91 (1.69)

L, large prostate (>60 g; $n=8$); S, small prostate (<40 g; $n=6$).

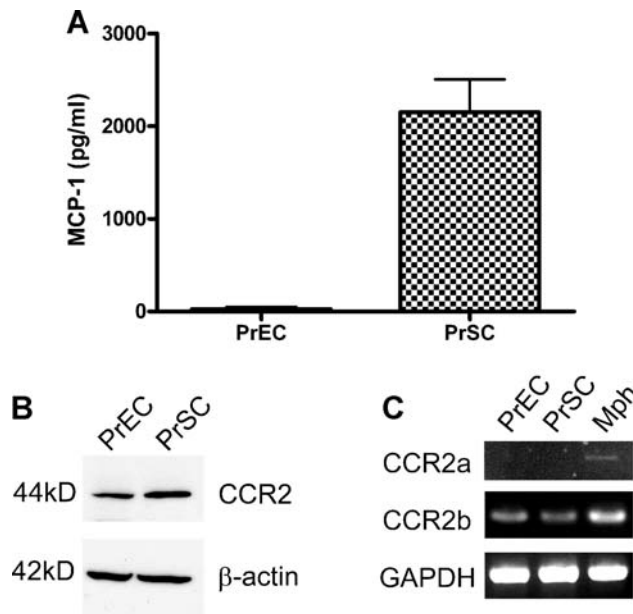


Fig. 2. Normal prostate epithelial and stromal cell lines secrete MCP-1 and express its receptor. **A:** MCP-1 in the supernatant of PrEC and PrSC by ELISA. PrEC and PrSC secrete 270 ± 40.7 and $2,150 \pm 615$ pg/ml MCP-1, respectively. **B:** CCR2 expression in both PrEC and PrSC by Western blot. **C:** RT-PCR analysis of CCR2 variants from PrEC and PrSC. Both cell lines express CCR2b but not CCR2a (Mph, macrophage).

completely inhibited this effect ($P < 0.05$; Fig. 3D). Monoclonal anti-MCP-1 neutralizing antibody also inhibited the growth stimulation of PrEC by PrSC-CM ($P < 0.05$; Fig. 3E).

PrSC were also subjected to MCP-1 inhibitors given the presence of MCP-1 receptor on their surface. Proliferation assays showed that only the highest concentration of RS102895 (10 μ M) inhibited PrSC proliferation, but that the monoclonal anti-MCP-1 antibody did not (data not shown).

MCP-1 Levels Correlate With Inflammatory Cytokines and Inflammation in BPH

IL-1 β , which appeared on the cytokine array data, is a pro-inflammatory molecule produced from monocyte/macrophages. MCP-1 also attracts T cells, and IFN- γ and IL-2, which are produced by activated T cells, are known to be upregulated 10-fold in BPH compared with normal prostate [17]. To test the effects of IL-1 β , IFN- γ , and IL-2 on MCP-1 secretion from PrEC and PrSC, these lines were incubated with 10 ng/ml of IL-1 β , 10 ng/ml of IFN- γ , and 10 U/ml of IL-2. PrEC secreted 286 ± 50 , $2,933 \pm 148$, and 37.1 ± 6.7 pg/ml of MCP-1 on stimulation by IL-1 β , IFN- γ , and IL-2, respectively, but only 16.0 ± 7.5 pg/ml of MCP-1 with-

out stimulation (Fig. 4A). PrSC secreted $1,888 \pm 631$ pg/ml of MCP-1 without stimulation, which increased to $6,084 \pm 131$, $3,818 \pm 212$, and $3,179 \pm 392$ pg/ml of MCP-1 on stimulation by IL-1 β , IFN- γ , and IL-2, respectively (Fig. 4B).

MCP-1 is known primarily as a chemotactic factor for monocyte/macrophages. We therefore examined the correlation of MCP-1 levels in EPS with the presence of the macrophage marker CD68 in those same specimens. Real-time PCR levels of CD68 mRNA in 25 available EPS samples correlated with MCP-1 levels from the same fluid ($r = 0.448$, $P < 0.05$; Fig. 5). There was statistically no correlation between gland weight and CD68 mRNA ($r = 0.169$, $P = 0.478$) or between gland weight and MCP-1 ($r = 0.364$, $P = 0.115$) in this limited set of 25 samples.

DISCUSSION

To date, much research on MCP-1 has been focused on its role in the progression of prostate cancer and in prostatic conditions other than benign prostatic hyperplasia. MCP-1 levels in EPS were found to be elevated in young men with CPPS IIIA and IIIB compared to a cohort of men with a variety of other benign prostatic pathologies including BPH [10]. In prostate cancer studies, MCP-1 has been shown to be a possible paracrine and autocrine factor in prostate cancer growth and migration [12,18], and anti-MCP-1 antibody has induced prostate cancer regression in vivo [14]. MCP-1 transcript is, however, expressed primarily by stromal smooth muscle cells and basal cells of the benign prostate [11]. Immunohistochemistry demonstrates a strong MCP-1 reaction in the fibromuscular stroma but not in the basal layer of these glands [11].

We found that the benign prostate stromal line PrSC secretes much higher levels of MCP-1 than the benign prostate epithelial line PrEC. However, PrEC can be induced to secrete high levels of MCP-1 by IL-1 β and particularly IFN- γ . Thus, primary sources of MCP-1 in the prostate may indeed be stromal cells and inflamed epithelial cells. We also observed that MCP-1 levels in EPS correlated with increasing prostate volume, and therefore hypothesized that MCP-1 secreted from stromal cells may stimulate the proliferation of prostatic epithelial cells. Both epithelial and stromal cells were found to express the MCP-1 receptor (CCR2). CCR2 has two alternatively spliced forms, CCR2a and CCR2b, which differ only in their carboxyl-terminal tails [19]. CCR2a is the major isoform expressed by mononuclear cells, while CCR2b is expressed by satellite cells, regenerative muscle fibers, and endometrium [20,21]. Prostatic epithelial and stromal cells were demonstrated to express primarily the CCR2b variant.

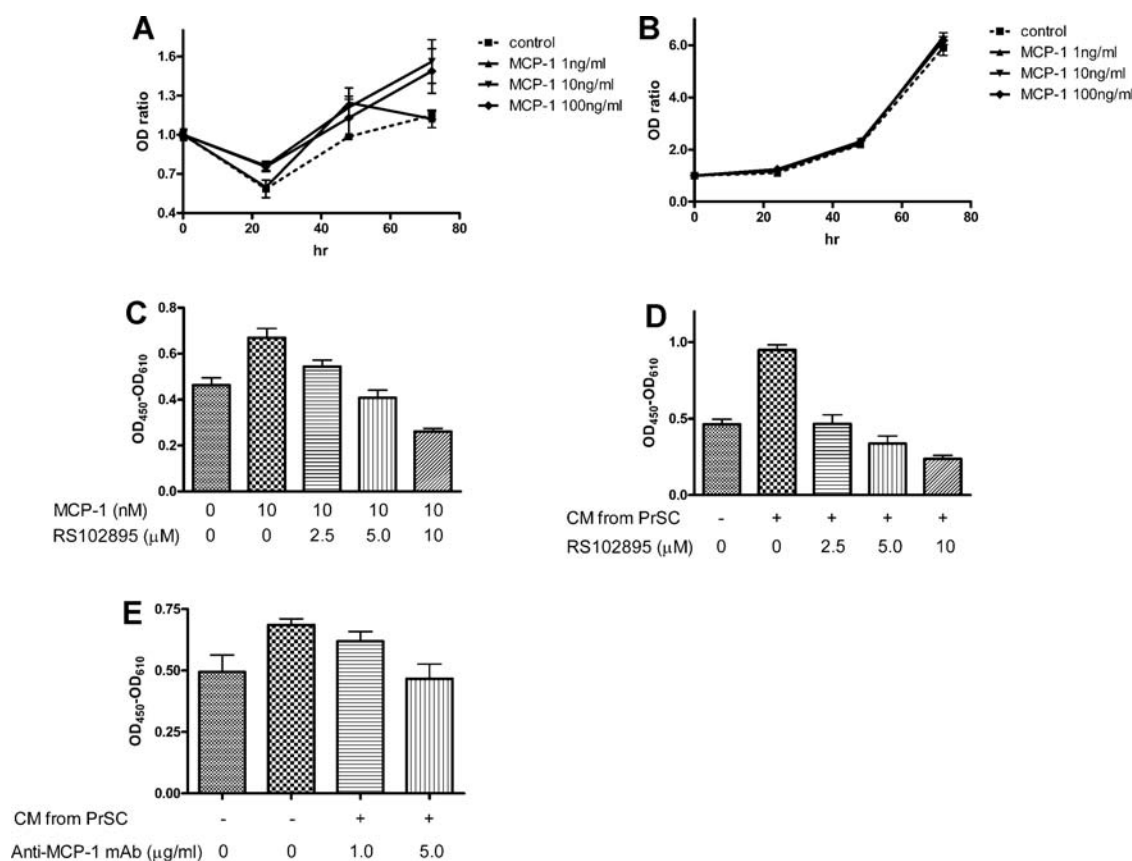


Fig. 3. MCP-I stimulates the proliferation of epithelial cells. WST-I proliferation assays showed that MCP-I stimulated the proliferation of PrEC in 72 hr significantly (10 and 100 ng/ml MCP-I: $P < 0.05$) (**A**) but not of PrSC (**B**). **C**: WST-I assay showed that 10 ng/ml MCP-I stimulated the growth of PrEC, and that RSI02895, a CCR2b antagonist, blocked this growth stimulation in a dose-dependent manner ($n = 4$; 5 μM , $P < 0.05$; 10 μM , $P < 0.01$). **D**: WST-I proliferation assays showed that conditioned media (CM) from PrSC stimulate the proliferation of PrEC ($P < 0.05$) and that RSI02895 suppresses this effect ($n = 4$; 5 and 10 $\mu\text{g/ml}$, $P < 0.01$). **E**: Monoclonal anti-MCP-I neutralizing antibody also suppresses the stimulation of PrEC growth by CM ($n = 4$; 10 $\mu\text{g/ml}$, $P < 0.05$).

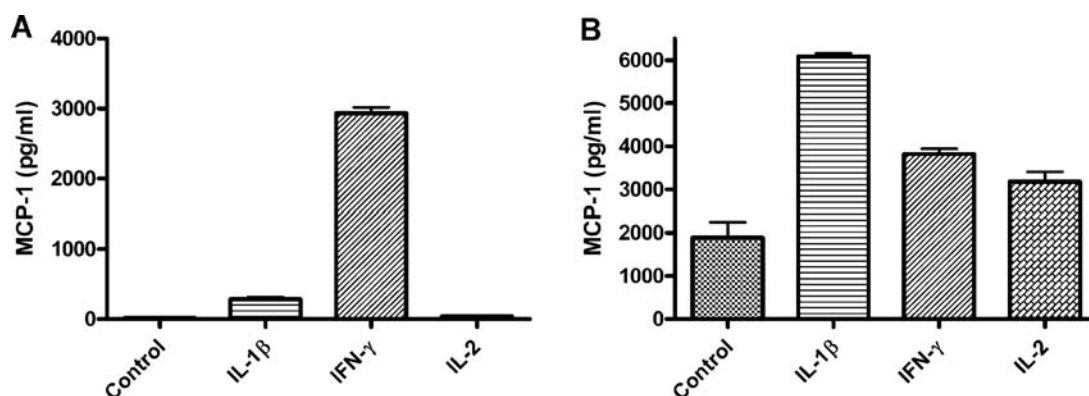


Fig. 4. Inflammatory cytokines enhance MCP-I secretion from prostatic epithelial and stromal cells. **A**: ELISA of culture media showed that PrEC secrete 286 ± 50 , $2,933 \pm 148$, and 371 ± 6.7 pg/ml of MCP-I upon stimulation by 10 ng/ml of IL-1 β , 10 ng/ml of IFN- γ , and 10 U/ml of IL-2, respectively, in contrast to 16.0 ± 7.5 pg/ml of MCP-I without any stimulation ($P < 0.05$). **B**: PrSC secreted $6,084 \pm 131$, $3,818 \pm 212$, and $3,179 \pm 392$ pg/ml of MCP-I upon stimulation by IL-1 β , IFN- γ , and IL-2, respectively, while PrSC secreted $1,888 \pm 631$ pg/ml of MCP-I without any stimulation ($P < 0.05$).

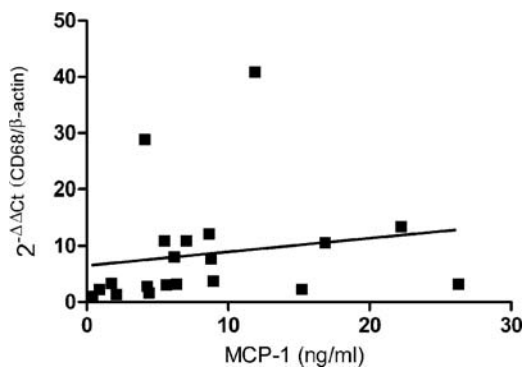


Fig. 5. Correlation between MCP-1 levels and macrophages in 25 EPS samples. CD68 mRNA levels normalized by β -actin were measured by real-time PCR and correlated with MCP-1 levels measured by ELISA ($r = 0.448$, $P < 0.05$)

Exogenous MCP-1 stimulated the proliferation of PrEC but not of PrSC, and conditioned medium from PrSC stimulated the growth of PrEC. While such medium contains MCP-1 it also likely contains numerous other growth factors such as KGF, IGF, and FGF, which play important roles in BPH development. However, when we added a specific MCP-1 antagonist or anti-MCP-1 antibody to the conditioned PrSC medium, we inhibited its growth effects on PrEC completely. These data strongly suggest that MCP-1 is an important regulator of prostatic epithelial proliferation, perhaps involved in conditions such as BPH. On the other hand, proliferation assays showed that only the highest concentration of RS102895 (10 μ M) inhibited the proliferation of PrSC, and that addition of monoclonal anti-MCP-1 antibody did not. This suggests a possible toxic effect of the antagonist molecule on PrSC rather than implying the existence of an autocrine MCP-1 pathway affecting PrSC. MCP-1 is known to promote the fibrosis in lung and kidney [22] and may stimulate collagen synthesis from prostatic stromal cells, but the effect of MCP-1 on PrSC remains to be determined.

MCP-1 is a chemotactic factor for lymphocytes and macrophages [23], immune cells that are often observed in BPH specimens. Typical infiltrates in BPH may consist of $\sim 70\%$ T lymphocytes, $\sim 15\%$ B cells, and $\sim 15\%$ macrophages as well as mast cells [24]. Our analyses showed a strong correlation between MCP-1 levels and CD68 mRNA levels in EPS. CD68 is a transmembrane glycoprotein, which is highly expressed by monocytes and macrophages and acts as a marker for them. Its association with MCP-1 levels suggests that the bioactive MCP-1 may be exerting a chemotactic role for monocyte/macrophages and those attracted monocyte/macrophages may secrete other factors to stimulate the proliferation of epithelial and stromal cells, though further studies would have to be performed to elucidate any causal relationship. CCR2

is expressed on activated and memory effector T cells [25,26], and MCP-1 is also a chemoattractant for T cells [27]. The effect of MCP-1 on T-cell differentiation remains controversial: MCP-1 stimulates Th2 polarization in the lymph node, whereas CCR2 activation stimulates Th1 polarization. In organ inflammation, MCP-1 acts to attract effector cells, which are abundant sources of IFN- γ [26].

IL-1 β has been found to be elevated in prostatic inflammation [28], and IFN- γ and IL-2 are elevated in BPH tissues [29]. IL-2 and IFN- γ may support fibromuscular growth in BPH [30]. In vitro, we noted that IL-1 β , IFN- γ , and IL-2 enhanced the secretion of MCP-1 from both epithelial and particularly from stromal cells. Surprisingly, the MCP-1 secretion from epithelial cells on stimulation by IFN- γ was almost as much as from stromal cells, although there was only trace MCP-1 secretion from epithelial cells without added cytokines.

An inflammatory stimulus and MCP-1 from stromal cells in the prostate may induce infiltration of T lymphocytes and macrophages, which in turn produce inflammatory cytokines. These cytokines might hypothetically stimulate the secretion of MCP-1 from stromal and epithelial cells, and secreted MCP-1 could in turn stimulate the growth of epithelial cells by a stromal-epithelial or autocrine interaction. Elevated MCP-1 levels in the prostate may also further attract inflammatory cells and contribute to a positive-feedback loop to stimulate BPH. Therefore, MCP-1 and its receptor molecule CCR2b in the prostate may present novel therapeutic targets for the treatment or prevention of BPH. Interestingly, BPH specimens have low macrophage inhibitory cytokine-1 (MIC-1) expression [31]. MIC-1 is an inhibitory factor for macrophage activation and thus acts in opposition to MCP-1 [32]. Thus, macrophages may be a key player in BPH development alongside T lymphocytes and other immunoeffectors.

High levels of MCP-1 exist in EPS, suggesting that MCP-1 levels in body fluids (prostatic secretions, urine, and serum) may potentially serve as markers for BPH, CPPS, and LUTS and help distinguish these conditions. MCP-1 and another monocyte/macrophage chemoattractant molecule macrophage inflammatory protein-1 α (MIP-1 α) have been proposed as possible biomarkers for CPPS, with intriguing data to support this [10]. In a careful study of EPS obtained in the clinic from a cohort of young male controls and CPPS patients, as well as from older men with BPH, elevated MCP-1 and MIP-1 α levels correlated with the CPPS diagnosis and were also slightly elevated in BPH. The MCP-1 levels in the EPS of men in our study were some 10-fold higher in those with large prostate glands, but this might be explained by different sampling

methodologies (ours represented EPS collected directly from ex vivo prostates rather than from clinic after digital rectal examination), different patient sets (our patients had prostates of known volume but unknown LUTS score, whereas in the Desireddi study neither prostate size nor LUTS score were mentioned), and the use of different commercial ELISA kits. Since there has been no biologic marker for BPH in clinical use to date, a molecule such as MCP-1 presents a novel, potentially important prognostic marker for prostate enlargement/BPH, and/or LUTS and associated conditions such as CPPS.

A limitation of our study is that the prostate glands from which prostatic secretions were expressed all contained small volume, low-grade prostate cancers. We chose radical prostatectomy specimens for study because they provide an excellent source of prostatic secretions uncontaminated by the remainder of the male urologic tract and they provide us with accurate pathologic weight, a reliable surrogate for prostate volume; these specific specimens were chosen because their predominant pathology was indeed BPH or benign prostatic tissue rather than cancer. Small, low-grade prostate cancers are in any case known to be present in the prostates of most men by their 7th decade, including those diagnosed only with clinical BPH [33]. Cancerous prostate glands have lower MCP-1 expression than benign prostate glands at the mRNA level [34]; thus, it appears unlikely that the presence of prostate cancer significantly elevated the MCP-1 levels in the EPS we studied.

CONCLUSIONS

In conclusion, increasing MCP-1 levels in EPS correlate with prostate volume as well as with the macrophage marker CD68. MCP-1 is expressed by prostatic stromal cells in vitro and stimulates the growth of prostatic epithelial cells. The inhibition of MCP-1 or of its receptor (CCR2) suppresses these stromal-epithelial interactions and results in the inhibition of prostate epithelial cell proliferation. Taken together, these data suggest that MCP-1 and macrophages may play a role in the pathogenesis of prostate enlargement, and that MCP-1 may be a potentially useful biomarker and therapeutic target for BPH.

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Immunomodulatory IL-18 binding protein is produced by prostate cancer cells and its levels in urine and serum correlate with tumor status

Kazutoshi Fujita, Charles M. Ewing, William B. Isaacs, and Christian P. Pavlovich

Brady Urological Institute, Johns Hopkins Medical Institutions, Baltimore, MD

Cytokines may play a role in the initiation and progression of prostate cancer. A cytokine antibody array was previously applied to prostatic fluid obtained from patients with prostate cancer, and interleukin 18 binding protein (IL-18BP), a potent inhibitor of interleukin 18, was noted to be significantly upregulated in cases with large volume disease. We sought to further characterize the association of IL-18BP with prostate cancer and determine whether IL-18BP levels in patient serum and urine samples had clinical relevance. IL-18BP was expressed and secreted by the prostate cancer cell lines DU145 and PC3 but not by LNCaP and CWR22, upon interferon- γ (IFN- γ) stimulation. IFN- γ -induced secretion of IL-18BP was enhanced by added TNF- α , IFN- α and IFN- β . The IL-18BP secreted from DU145 and PC3 functionally inhibited IL-18. Immunohistochemical analyses showed positive IL-18BP staining in prostate cancer cells as well as in macrophages in radical prostatectomy specimens. Significant differences in urinary IL-18BP levels (normalized by total protein) collected post-DRE were found between cases with and without cancer on biopsy ($p = 0.02$) and serum IL-18BP levels correlated with Gleason score ($p = 0.03$). Our finding of elevated IL-18BP secretion from prostate cancer cells suggests an attempt by cancer to escape immune surveillance. IL-18BP merits further study as a marker of aggressive prostate cancer and as a therapeutic target.

Chronic inflammation is commonly observed in radical prostatectomy specimens, and prostate tissues often contain increased inflammatory infiltrates, including T cells, B cells, macrophage, neutrophils and mast cells.^{1,2} The immune system can specifically identify and eliminate tumor cells: Tumor infiltration by T cells, NK cells and/or NKT cells is associated with an improved prognosis for a number of different tumor types.^{3,4} However, the immune system has a paradoxical role in tumor development, as it has been established that chronic activation of innate immune cells, such as macrophages, mast cells and neutrophils, contributes to cancer development.⁴ Tumor cells may also create an immunosuppressive environment in cancer patients, and may escape immune surveillance through various mechanisms.⁵ Cytokines secreted by tumor and inflammatory/immune cells are one factor that can promote tumor development and tumor cell survival in an otherwise immunologically intact host.³

We previously applied a human cytokine array to search for cytokines in prostatic fluid that may be associated with aggressive prostate cancer and found that interleukin 18 binding protein (IL-18BP) was elevated in cases with large volume disease.⁶ IL-18BP is a secreted glycoprotein possessing high-affinity binding and an ability to neutralize interleukin-18 (IL-18).⁷ IL-18 in turn is a mediator of T_H1 cytokines, induces high levels of interferon gamma (IFN- γ) secretion by NK cells, CD8⁺ T cells and T_H1 cells, potentiates IL-12-induced T_H1 development and plays an important role in T-cell proliferation.⁸ It also enhances FasL-mediated cytotoxicity of NK cells, CD8⁺ T cells and T_H1 cells and has proinflammatory properties such as inducing macrophage chemotactic molecules. In mice, IL-18 exerts its antitumor activity *via* IFN- γ , NK cells, CD8⁺ T cells and CD4⁺ Fas-dependent cytotoxicity.⁸ The IL-18 binder IL-18BP is thought to form part of a negative feedback loop designed to limit T_H1 immune activation. IL-18BP is constitutively expressed in human spleen, leukocytes, monocytes, and endothelial cells.⁷ IL-18BP expression can also occur in keratinocytes, renal mesangial cells and colon cancer epithelial cells upon induction by IFN- γ .⁹⁻¹¹ In our study, we sought to further characterize the association of IL-18BP with prostate cancer, using prostate cancer tissues and cell lines, and assess whether its presence in patient serum and urine samples had clinical relevance.

Key words: prostate cancer, IL-18 binding protein, urinary marker
Grant sponsor: NIH/NIDDK; **Grant number:** 1K23DK071262;
Grant sponsor: Department of Defense; **Grant number:** PC041214;
Grant sponsor: NIH/NCI; **Grant number:** U24 CA115102
DOI: 10.1002/ijc.25705
History: Received 1 May 2010; Accepted 13 Sep 2010; Online 28 Sep 2010

Correspondence to: Christian P. Pavlovich, The Brady Urological Institute, Johns Hopkins Bayview Medical Center, 301 Building, Room 3104, 4940 Eastern Ave., Baltimore, Maryland 21224, USA, Tel.: 410-550-0013, Fax: 410-550-3341, E-mail: cpavlov2@jhmi.edu

Material and Methods

Cell culture

Cell lines. LNCaP, PC3, DU145, CWR22 and KG-1 were obtained from American Type Culture Collection (Rockville,

MD). The cells were maintained in RPMI supplemented with 10% heat-inactivated FBS and 1% penicillin/streptomycin at 37°C containing 5% CO₂.

Human sample collection

All samples were collected using IRB-approved protocols. Frozen prostate tissues were obtained from an institutional tumor bank from preserved radical prostatectomy specimens. The prostate cancer tissues assessed consisted of Gleason 6: $n = 4$, Gleason 7: $n = 1$, Gleason 8: $n = 1$, and Gleason 9: $n = 1$ cancers. Six benign areas from the radical prostatectomy specimens were also obtained and assessed. Urine samples were collected in the Urology Clinic. Initial voided urine specimens (10 to 100 ml) were prospectively collected from 99 men with an indication for prostate biopsy immediately after digital rectal examination (DRE) during a single office visit. Of the 99 men who provided urine for analysis, 67 were found to have prostate cancer (median age (range): 60 (45–84), median PSA (range): 5.05 ng/ml (1.7–20.5), suspicious DRE: 19.4%, Gleason score 6: $n = 43$, Gleason score 7: $n = 21$, Gleason score 8: $n = 1$, Gleason score: $n = 2$), and 32 were negative on biopsy (median age (range): 62 (40–81), median PSA (range): 5.4 ng/ml (0.6–11.5), suspicious DRE: 18.7%). Voided urine specimens were kept at 4°C for up to 4 hr prior to centrifugation for 10 min at 1,000 g to remove sediments and then urine supernatants were kept at –80°C until analysis. In addition, 79 archival serum samples were obtained from our biorepository and linked to information about patient prostate health status and other relevant demographic and pathologic data. Of the 79 sera analyzed, 69 came from men who had prostate cancer (median age (range): 61 (47–69), median PSA (range): 5.3 ng/ml (0.9–27.8), suspicious DRE: 24.3%, Gleason score 6: $n = 27$, Gleason score 7: $n = 35$, Gleason score 8: $n = 5$, Gleason score: $n = 2$), and 10 came from men without known prostate cancer and at low risk for the disease (median age (range): 53 (46–64), median PSA (range): 1.2 ng/ml (0.5–1.9), suspicious DRE: 0%). Of the 69 men with prostate cancer, 30 had organ-confined disease (median age (range): 57.5 (48–66), median PSA (range): 4.7 ng/ml (2.9–13.4), suspicious DRE: 13.3%, Gleason score 6: $n = 17$, Gleason score 7: $n = 13$, Gleason score 8: $n = 0$, Gleason score: $n = 0$) and 39 had nonorgan-confined disease (median age (range): 62 (47–69), median PSA (range): 6.5 ng/ml (0.9–27.8), suspicious DRE: 32.5%, Gleason score 6: $n = 10$, Gleason score 7: $n = 22$, Gleason score 8: $n = 5$, Gleason score: $n = 2$).

Reverse-transcriptase PCR

Total RNA was extracted from LNCaP, CWR22, PC3 and DU145 after 24 hr incubation with or without 10 ng/ml IFN- γ or from frozen prostate tissues with the RNeasy Mini Kit (Qiagen, Valencia, CA). Total RNA (1 μ g) was treated with DNase I (1U, Invitrogen, Carlsbad, CA) for 15 min. These tissues were selected from frozen radical prostatectomy specimens: For prostate cancer, areas were selected for >80%

tumor content by light microscopy of representative sections, and noncancerous areas were selected from adjacent benign prostatic tissue. First-strand cDNA was produced with random hexamers as per the manufacturer's recommendations (Omniscript RT kit/Qiagen). PCR amplification of *IL-18BP*, *GAPDH*, *JAK1* and *JAK2* was done with HotStar Taq Plus Master Mix (Qiagen). Primers used were as follows: *IL-18BP*, 5'-ATGGAACGCTGAGCTTATCCT-3' (forward) and 5'-GGCCCTGTGCTGAGTCTTA-3' (reverse); *GAPDH*, 5'-ACCAGGGCTGCTTTTAACTCT-3' (forward) and 5'-GATGACAAGCTTCCCGTTCT-3' (reverse); *JAK1*, 5'-GGCGTCATTCTCCAAAGAAGC-3' (forward) and 5'-TCAACAGAAACAACATTGTTGGT-3' (reverse); *JAK2*, 5'-AACCTCACAACATTACAGAG-3' (forward) and 5'-GATTTCCTGTC TTCCTGTCTT-3' (reverse). Amplification conditions were as follows: 15 min at 95°C (one cycle) and 45 sec at 94°C; 45 sec at the annealing temperature (60°C for *IL-18BP* and *GAPDH*, 54°C for *JAK1* and 50°C for *JAK2*); and 60 sec at 72°C (32 cycles for *IL-18BP* and *GAPDH* and 35 cycles for *JAK1* and *JAK2*) and 72°C for 5 min (one cycle).

Western blot analysis

After washing with ice-cold PBS, cells were harvested in RIPA buffer (Thermo Scientific, Rockford, IL) supplemented with Halt protease inhibitor cocktail (Thermo Scientific). Total cellular protein concentrations were determined by using a BCA protein assay reagent (Thermo Scientific). 60 μ g protein of lysates were subjected to SDS-PAGE under the reducing condition, and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). Membranes were immunoblotted with mouse monoclonal antihuman IL-18BP antibodies at a dilution of 1:250 (MAB1191, R&D Systems, Minneapolis, MN) followed by horseradish peroxidase-conjugated secondary antibodies, and developed with the Super Signal West Dura Extended Duration Substrate kit (Thermo Scientific). A total of 30 μ g protein of lysates of LNCaP, CWR22, PC3 and DU145 cells treated with or without 100 ng/ml IFN- γ for 15 min were also subjected to SDS-PAGE. After Western transfer, membranes were immunoblotted with antibodies for phospho-STAT1 (#9171, Cell Signaling Technology, Danvers, MA), STAT1 (#9172, Cell Signaling Technology) or beta actin and developed as mentioned above.

Measurement of IL-18BP in cell culture conditioned media

2×10^5 of LNCaP, CWR22, PC3 and DU145 cells were separately seeded in 24-well plates and 24 hr later media were changed to media containing 0, 0.4, 2.0, 10, 50 ng/ml of IFN- γ (285-IF, R&D Systems). In another set of experiments, 2×10^4 of PC3 and DU145 were separately seeded in 96-well plates (Falcon), and after incubation for 24 hr at 37°C, media were changed to media containing 200 ng/ml IL-12 (219-IL, R&D Systems), 200 ng/ml TNF- α (210-TA, R&D Systems), 10,000 IU/ml IFN- α (11,200, R&D Systems) and 5,000 IU/ml IFN- β (11,415, R&D Systems) with or without 10 ng/ml IFN- γ . After 24 hr of incubation, plates from both

sets of experiments were centrifuged at 1,000 g for 10 min, and supernatants were collected and subjected to ELISA.

Enzyme-linked immunosorbent assay (ELISA)

IL-18BP in cell culture supernatants, urine and sera, IFN- γ in cell culture supernatants, and IL-18 in sera were measured by ELISA. A DuoSet ELISA development system (R&D Systems) was used to detect IL-18BP (IL18BP primary isoform α) and IFN- γ . A human IL-18 ELISA kit (MBL, Nagoya, Japan) was used to detect IL-18 in serum. Each cytokine was measured based on the manufacturer's recommendations. Urine and serum samples were assayed at a 10-fold and 4-fold dilution, respectively. ELISA data from urine samples were normalized by total urinary protein levels as measured by Dade Dimension RxL (Siemens Healthcare Diagnostics, Deerfield, IL). Cell supernatant and serum ELISA data were not normalized. Free IL-18 levels were calculated according to the law of mass action as previously described,¹² and the dissociation constant (Kd) was 0.4 nM.

IL-18BP biological assay

LNCaP, CWR22, DU145 and PC-3 were seeded to 75 cm² flasks and stimulated by 0 or 10 ng/ml IFN- γ for 24 hr. Then, these cultures were washed three times with HBSS in the same flasks, and incubated with 10% RPMI/FBS for 2 days. 1.2×10^6 KG-1 cells were incubated with 100 μ l of conditioned media from these cultures and with 20 ng/ml TNF- α and 40 ng/ml IL-18 at 37°C. After 24 hr incubation, supernatants from the KG-1 cell cultures were collected and subjected to IFN- γ ELISA.

Immunohistochemical analysis

A total of 5 μ m paraffin-embedded radical prostatectomy sections were subjected to immunohistochemistry, performed with the PowerVision+ IHC Detection System (Vision BioSystems, Norwell MA) according to the manufacturer's recommendations. The sections were deparaffinized and rehydrated, and after the slides were steamed for 40 min in Target Retrieval Solution (DakoCytomation, Carpinteria, CA) for antigen retrieval, endogenous peroxidase activity was blocked with 3% H₂O₂. Slides were incubated with mouse antihuman IL-18BP monoclonal antibody (MAB119, RND) at a dilution of 1:50 in PBS overnight at 4°C. Staining was visualized using 3,3'-Diamino-benzidine (DAB) (FAST 3,3'-Diamino-benzidine Tablets, Sigma, Saint Louis, MO, USA, FAST 3,3'-Diamino-benzidine Tablets) and slides were counterstained with hematoxylin. Human Tonsil was used as the positive control.

Data analysis and statistics

Results were expressed as mean \pm SD. Statistical analyses were done using GraphPad Prism 4.0 for Windows. Mann-Whitney tests and Student's *t*-tests were used to analyze the difference of two categories in clinical samples (prostatic fluids, urine and serum) and *in vitro* experiments, respectively. Statistical significance was defined as a *p* value < 0.05.

Results

Prostate cancer cell lines express IL-18BP

We analyzed *IL-18BP* mRNA expression in prostate cancer cell lines by RT-PCR (Fig. 1a). PC3 strongly expressed *IL-18BP* mRNA, DU145 expressed it moderately, and LNCaP and CWR22 expressed far less. Western blots of lysates of PC3 and DU145 revealed an approximately 42 kDa band detected by monoclonal antibody against human IL-18BP α , consistent with the size of human IL-18BP α . This band was not detected in cell lysates from LNCaP or CWR22 (Fig. 1b). Since IL-18BP is a secretory protein, its levels in supernatants of LNCaP, CWR22, PC3 and DU145 were examined by ELISA. Since IFN- γ induces IL-18BP expression in monocytes and nonleukocytic cells,^{9,10,13} we stimulated prostate cancer cell lines with IFN- γ to examine IL-18BP secretion. 24 hr after IFN- γ stimulation, IL-18BP protein was detected in supernatants from PC3 and DU145 cell lines (but not in those from LNCaP or CWR22) in a dose-dependent manner (Fig. 1c). IL-18BP is induced from human peripheral mononuclear cells not only by IFN- γ but also by IL-12.¹³ We examined the effect of this other cytokine, IL-12, as well as of TNF- α , IFN- α and IFN- β on secretion of IL-18BP from prostate cancer cell lines. No cytokine other than IFN- γ induced IL-18BP secretion from PC3 and DU145, but TNF- α , IFN- α , IFN- β enhanced IFN- γ -induced secretion of IL-18BP from DU145, and TNF- α enhanced its secretion from PC3 (*p* < 0.01) (Fig. 1d). No combination of these cytokines stimulated release of IL-18BP from LNCaP or CWR22 cells (data not shown).

To further study the variable response of some prostate cancer cell lines to IFN- γ stimulation with respect to IL-18BP secretion, the expression of IL-18BP α mRNA was also assessed 24 hr after IFN- γ stimulation. IL-18BP α mRNA was highly upregulated in both PC3 and DU145 but only marginally upregulated in LNCaP and CWR22 (Fig. 2a). The poor response of LNCaP and CWR22 to IFN- γ stimulation at the messenger level may be the reason these lines do not secrete significant amounts of IL-18BP α . IFN- γ is known to exert its effect mainly through the Janus kinases JAK1/JAK2 which lead to STAT1 activation.¹⁴ Phosphorylated STAT1 then acts directly on the proximal site of the IL18BP promoter which is crucial for IL18BP induction.¹⁵ The expression of JAK1 and JAK2 was examined in these prostate cancer cell lines by RT-PCR: LNCaP and CWR22 expressed very little JAK1 compared to PC3 and DU145, whereas JAK2 expression did not differ between cell lines (Fig. 2b). Prostate cancer cell lines were treated with or without IFN- γ and cell lysates were immunoblotted with antibodies for STAT1 and phosphorylated STAT1. PC-3, DU145 and LNCaP cells had baseline STAT1 expression, and CWR22 had slight STAT1 expression. With IFN- γ treatment, both PC-3 and DU145 cells exhibited strong STAT1 phosphorylation, whereas LNCaP had only a trace STAT1 phosphorylation signal and CWR22 had none (Fig. 2c). Low JAK1 expression and unphosphorylated STAT1 are reasonable explanations for the inability of LNCaP and CWR22 to secrete IL-18BP.

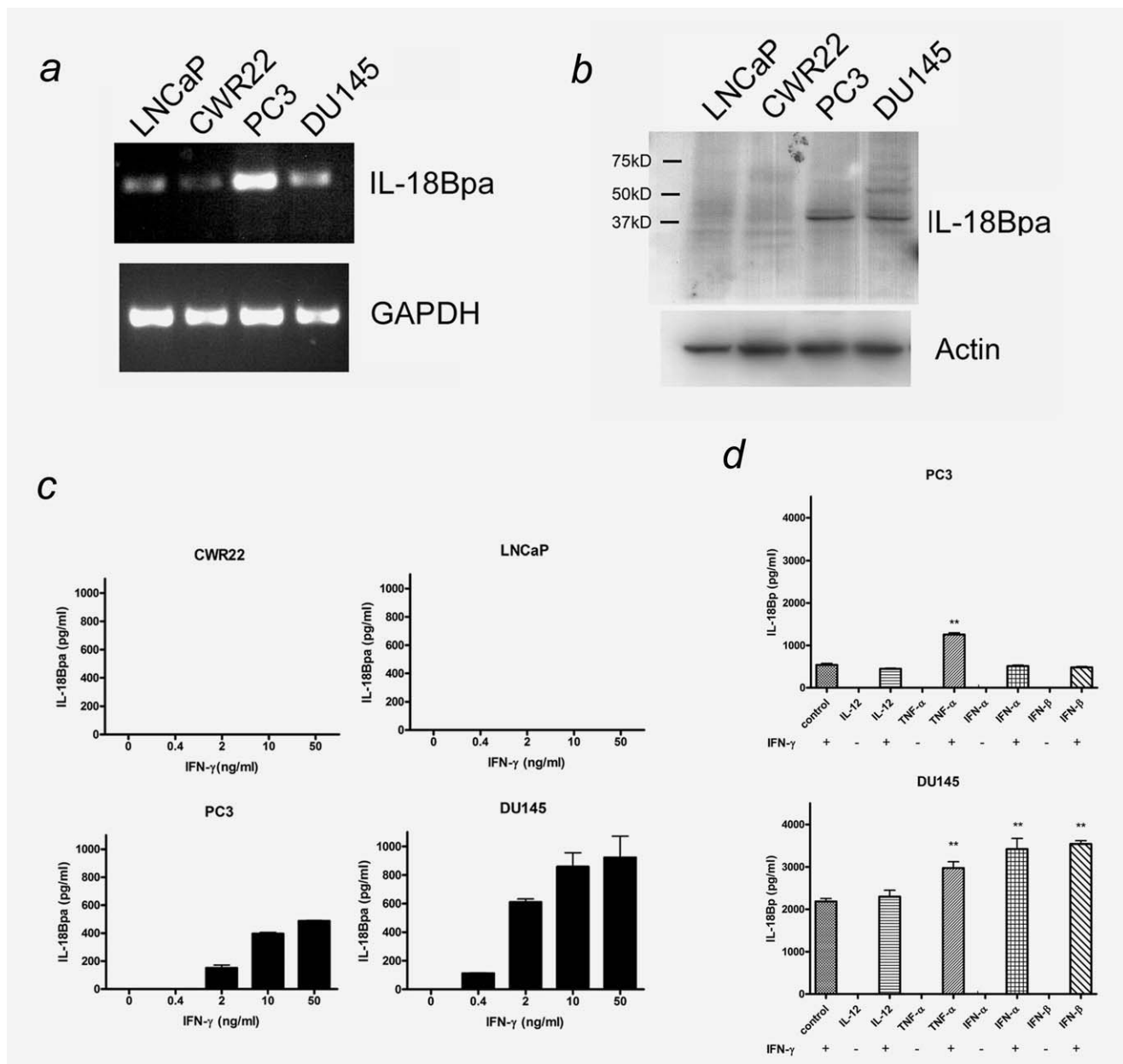


Figure 1. IL-18BP expression in prostate cancer cell lines. (a) RT-PCR analysis of IL-18BP expression from prostate cancer cell lines. (b) IL-18BP expression by Western blot for four prostate cancer cell lines. (c) IL-18BP secretion from prostate cancer cell lines. IL-18BP was measured in the supernatants of LNCaP, CWR22, PC3 and DU145 after 24 hr-stimulation with IFN- γ (0, 0.4, 2.0, 10, 50 ng/ml) by ELISA ($n = 3$). (d) PC3 (upper panel) and DU145 (lower panel) were incubated with 200 ng/ml IL-12, 200 ng/ml TNF- α , 10,000 IU/ml IFN- α or 5,000 IU/ml IFN- β with or without 10 ng/ml IFN- γ for 24 hr ($n = 3$), and then IL-18BP in supernatants were measured by ELISA. (** $p < 0.01$ compared to control).

The secreted IL-18BP from prostate cancer cell lines appears to be biologically active in vitro

To confirm that the IL-18BP secreted from PC3 and DU145 was biologically active, conditioned media from LNCaP, CWR22, PC3 and DU145 with 0 ng/ml or 10 ng/ml IFN- γ were incubated with KG-1 cells (which secrete IFN- γ secondary to stimulation by IL-18 and TNF- α in IL-18 and TNF- α .

KG-1 cells incubated with conditioned media from IFN- γ -stimulated LNCaP and CWR22 (together with IL-18 and TNF- α), did not have their IFN- γ secretion suppressed, but the secretion of IFN- γ from KG-1 cells was suppressed by conditioned media from IFN- γ -stimulated PC3 and DU145 even in the presence of high levels of exogenous IL-18 and TNF- α (Fig. 2d). The concentrations of IL-18BP in

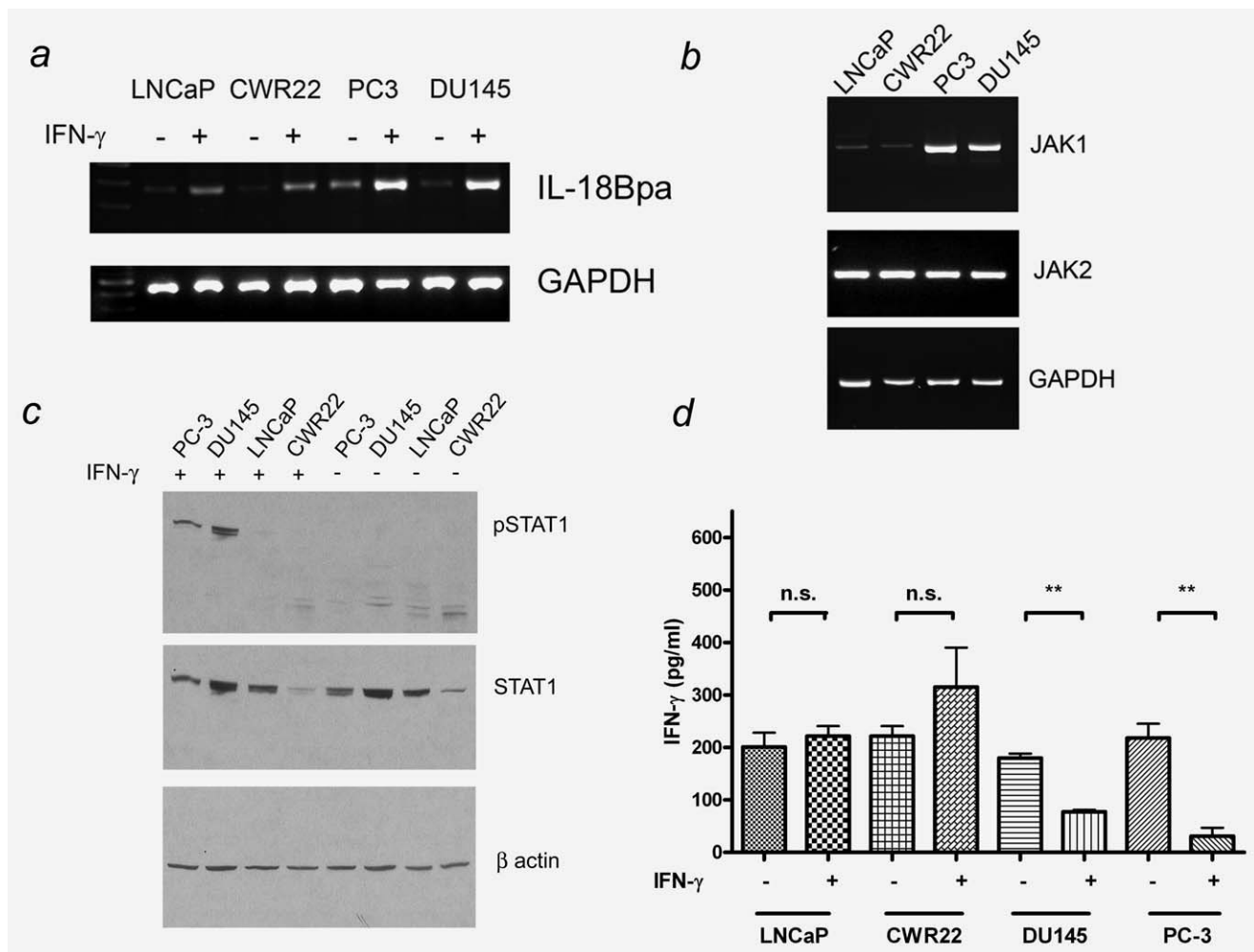


Figure 2. (a) RT-PCR analysis of *IL-18BP* expression in prostate cancer cell lines after the 24 hr stimulation with 10 ng/ml IFN- γ . (b) RT-PCR analysis of *JAK2* expression in prostate cancer cell lines. (c) Phosphorylation of STAT1 in prostate cancer cell lines. Total cell lysates from prostate cancer cell lines with or without IFN- γ were immunoblotted using antibodies for phosphorylated STAT1 (pSTAT1) (upper panel), STAT1 (middle panel) and β -actin (lower panel). (d) IFN- γ -conditioned media (0 or 10 ng/ml) from prostate cancer cell lines and control media were used to resuspend KG-1 cells with TNF- α (20 ng/ml) and IL-18 (40 ng/ml). After a 24-hr incubation period, IFN- γ production from KG-1 cells was measured by ELISA ($n = 3$). Means \pm SD are shown; ** $p < 0.01$ (unpaired t -test).

conditioned media of PC3 and DU145 were 6.66 ± 0.41 and 10.0 ± 0.52 ng/ml, respectively. IL18BP was not detected in conditioned media from LNCaP and CWR22. These data suggest that IFN- γ -stimulated PC3 and DU145 secrete biologically active IL-18BP that suppresses IL-18-induced production of IFN- γ from KG-1 cells.

IL-18BP is expressed by prostate tissues

RT-PCR analysis showed that IL-18BP transcript is expressed by prostate cancer from radical prostatectomy specimens and also from benign areas of prostates with prostate cancer (Fig. 3a). Expression analysis using the Oncomine Cancer Microarray database (<http://www.oncomine.org>)¹⁶ shows that IL-18BP expression is significantly increased in lymph node metastases of prostate cancer ($n = 6$)

compared to nonmetastatic prostate cancer cases ($n = 63$) ($p < 0.01$) (Fig. 3b).

IL-18BP is strongly expressed by monocytes/macrophages¹³ but is not known to be expressed by prostate cancer. To better define the sources of IL-18BP expression in the prostate, we analyzed prostate specimens from patients with prostate cancer immunohistochemically. Tonsil was used as a positive control, where macrophages stain by antihuman IL-18BP monoclonal antibody (Fig. 3c i). In prostate specimens, prostate cancer epithelial cells were positive and benign prostatic epithelial cells were negative for IL-18BP, but in some of the glands with inflammation, positively staining macrophages were found inside and around the glands (Fig. 3c ii-iv). Thus, both prostate cancer cells and macrophages appear to be sources of IL-18BP in cancerous prostate glands.

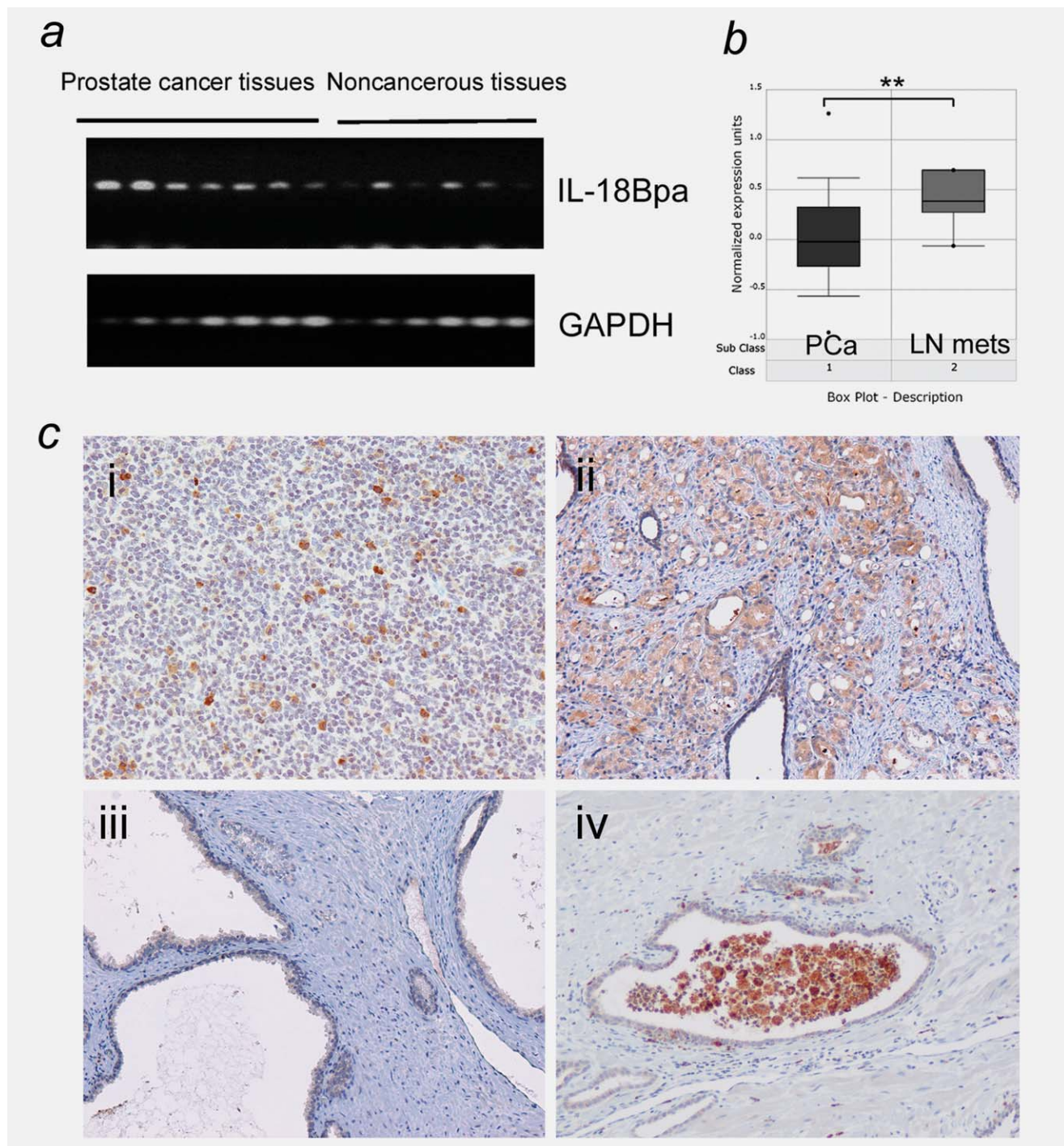


Figure 3. (a) RT-PCR analysis of *IL-18BP* expression in prostate cancer ($n = 7$) and noncancerous prostate tissue harvested from frozen radical prostatectomy specimens ($n = 6$). (b) *IL-18BP* expression in prostate cancer (PCa, $n = 63$) and lymph node metastatic prostate cancer (LN mets, $n = 6$) from the Oncomine Cancer Microarray Database (<http://www.oncomine.org>)¹⁶ (** $p < 0.01$). (c) Immunohistochemical analysis of *IL-18BP* expression with mouse monoclonal antihuman *IL-18BP* antibody (i) Tonsils. (ii) Prostate cancer (Gleason score 9). (iii) Normal glandular epithelium from the same slide as Fig.3c ii. (iv) Glands with inflammation filled with macrophages from another radical prostatectomy specimen.

IL-18BP levels in urine after DRE and serum

We analyzed *IL-18BP* levels both in the urine after prostatic manipulation (DRE) and in serum, to examine any correla-

tions between *IL-18BP* and prostate cancer. Significant differences in post-DRE urinary *IL-18BP* levels (normalized by total protein—TP) were found between cases with and

without cancer on biopsy ($p = 0.015$) (Fig. 4a). The area under the receiver operating characteristics curve was 0.658 for IL-18BP/TP (95% CI 0.5461–0.7692, $p = 0.011$) versus 0.502 for PSA (95% CI 0.3741–0.630, $p = 0.975$) in this cohort (Fig. 4b). The sensitivity for cancer detection was 69% and the specificity was 56%. In contrast, there was no significant difference in serum IL-18BP levels between normal cases ($n = 10$) and cases with prostate cancer ($n = 69$). However, serum IL-18BP levels from cases with high Gleason sum

(7 or more) were elevated compared to those of low Gleason score (6 or less) ($p = 0.029$) (Fig. 4c). Serum IL-18 levels were also measured but levels of this cytokine did not correlate with cancer status or Gleason score (Fig. 4c). Free IL-18 levels were also calculated (normal: 4.6 ± 2.5 pg/ml, organ confined: 4.4 ± 1.9 pg/ml, nonorgan confined: 5.1 ± 2.8 pg/ml, low Gleason: 5.0 ± 2.2 pg/ml, high Gleason: 4.5 ± 2.6 pg/ml); These did not statistically correlate with cancer status or Gleason score.

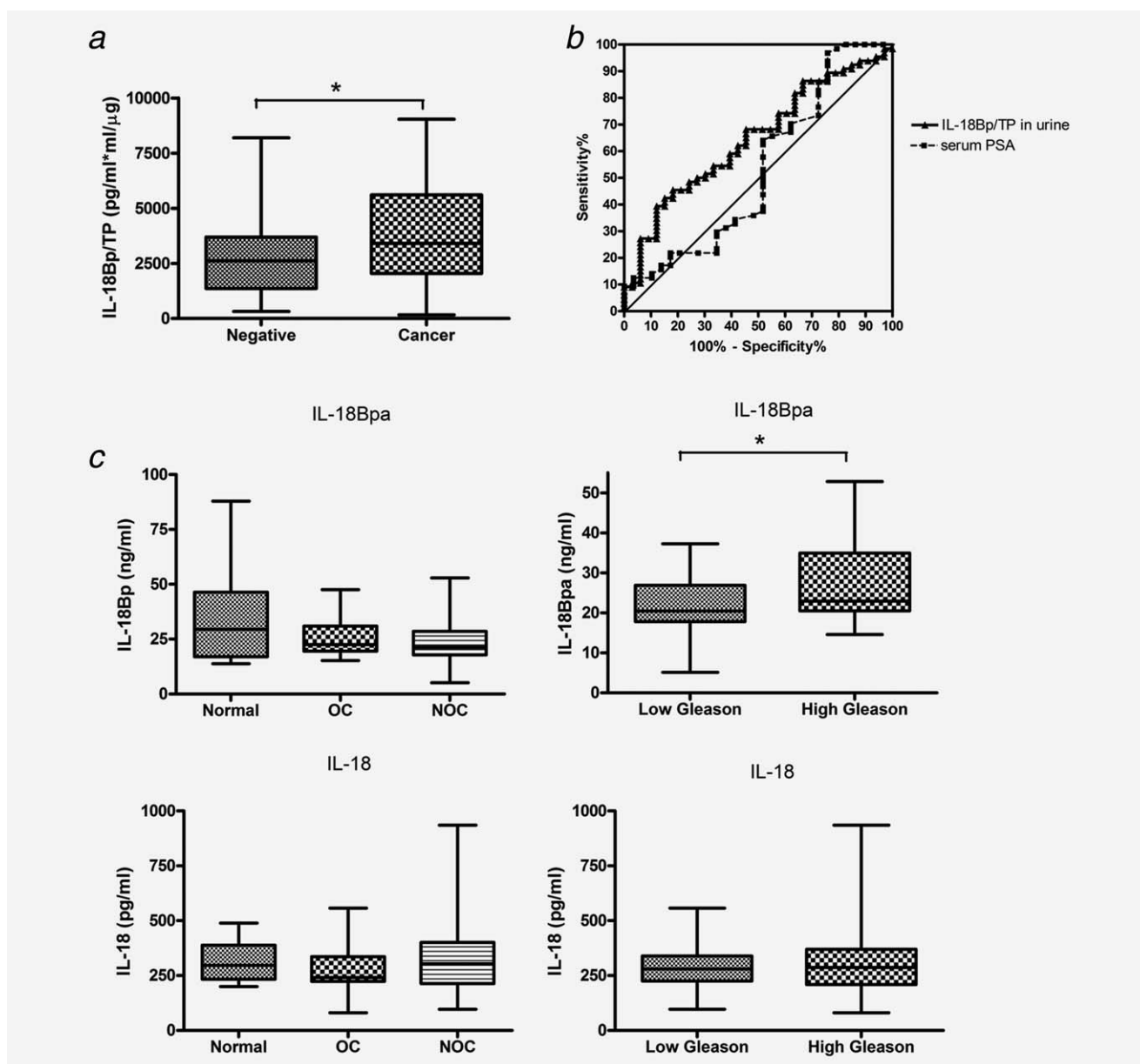


Figure 4. IL-18BP levels in urine after DRE and in serum. (a) Post-DRE urinary IL-18BP levels normalized by TP in cases with cancer (Cancer, $n = 67$) and without cancer on biopsy (Negative, $n = 32$). (b) The area under the receiver operating characteristic curve was 0.658 for IL-18BP/TP (95% CI 0.5461–0.7692, $p = 0.011$) versus 0.502 for PSA (95% CI 0.374–0.630, $p = 0.975$) in this cohort. (c) IL-18BP (upper panel) and IL-18 (lower panel) levels in serum. Key: Normal ($n = 10$), OC: organ-confined disease ($n = 30$), NOC: nonorgan-confined disease ($n = 39$), Low Gleason: 6 or less, High Gleason: 7 or more ($*p < 0.05$).

Discussion

We demonstrate for the first time that prostate cancer cell lines contain IL-18BP transcripts and protein and that this IL-18 inhibitor is secreted in a bioactive form by the prostate cancer cell lines PC3 and DU145. In addition, IL-18BP is present in prostate cancer cells and macrophages from radical prostatectomy specimens by immunohistochemistry analysis. Finally, using clinically obtained biofluids, significant differences in urinary IL-18BP levels between cases with and without cancer on biopsy were found using urine collected after prostate examination (DRE), and serum IL-18BP levels correlated with Gleason score.

IL-18BP is a member of the Ig superfamily and resembles the extracellular segment of cytokine receptors (but is distinct from the IL-1 and IL-18 receptor family).⁷ Human IL-18BP has 4 isoforms, a, b, c and d, derived from mRNA splice variants.¹⁷ Isoform a, the most abundant isoform, exhibits the greatest affinity for IL-18 and neutralizes it, while type c has 10-fold weaker affinity.^{7,17} In contrast, isoforms b and d lack the ability to neutralize IL-18. IL-18BP has N-linked glycosylation sites, and the band sizes of isoforms a, b, c and d on Western blot are 42, 16, 40 and 35 kDa, respectively.¹¹ PC3 and DU145 each showed a single band on Western blot at ~ 42 kDa; therefore the main IL-18BP isoform found in prostate cancer cell lines appears to be isotype a (IL-18BP_a). These two cell lines (unlike LNCaP and CWR22) were also shown to contain the relevant intracellular machinery (JAK1 and JAK2) to respond to IFN- γ induction of the IL-18/IL-18BP pathways.

The secretion of IL-18BP by prostate cancer may dampen the antitumor effect of immune surveillance by IL-18. IFN- γ is produced by NK cells, NKT cells and T cells upon IL-12 and IL-18 stimulation; the antitumor effect of IFN- γ has been observed in many animal models.^{18,19} IFN- γ can exert both direct antiproliferative and antimetabolic effects on tumor cells, and inhibit angiogenesis within a tumor.²⁰ IFN- γ also activates NK cells and NKT cells against tumors, enhances MHC class I expression on tumor cells, and stimulates CTL activation and T_H1 cell differentiation.¹⁸ In our experiments, IL-18BP_a was secreted from prostate cancer cells in response to IFN- γ stimulation. IL-18BP was not detected from PC3 and DU145 without stimulation by IFN- γ , but IL-18BP may be constitutively secreted from these cell lines at concentrations below the ELISA detection limit. IL-18BP secretion from prostate cancer cells was also enhanced by TNF- α , IFN- α and IFN- β , although the concentrations used in order to enhance IL-18BP secretion *in vitro* were higher than those found in serum. Daily administration of 1×10^7 IFN- α was reported to enhance plasma IL-18BP levels in humans.²¹ Several poxviruses encode an IL-18BP homologous protein that inactivates host-derived IL-18 and inhibits NK cell responses to escape host immune surveillance.²² Prostate cancer may also utilize IL-18BP to escape immune surveillance mechanisms, suggesting that IL-18BP may be a possible

target for cancer treatment. Recently, the use of recombinant human IL-18 to treat advanced cancers was studied, and 2/28 patients experienced partial responses.^{23,24} The administration of rhIL-18 induced IL-18BP in these patients, as well as IFN- γ and GM-CSF. It is tempting to speculate whether inhibition of IL-18BP induction might have improved these outcomes.

In our study, increased serum IL-18BP levels correlated with increasing prostate cancer Gleason score. Others have previously demonstrated that increased IL-18 levels, assessed immunohistochemically, correlate with favorable outcomes in prostate cancer.²⁵ We therefore also measured serum IL-18, but found no correlation with cancer status and Gleason score. IL-18BP tightly binds to IL-18 and blocks its activity. Free IL-18 could exert biologic activity, and its levels could be calculated knowing IL-18BP levels, IL-18 levels and the K_d (0.4 nM).¹² Calculated free IL-18 levels in various diseases were reported and have been related to various disease states.^{21,26} In our study, calculated free IL-18 levels correlated with neither cancer status nor Gleason score. Nevertheless, investigating IL-18BP, total IL-18 and free IL-18 levels in serum from prostate cancer cases may yield interesting findings given a larger study cohort. The data from the Oncomine Cancer Microarray Database (<http://www.oncomine.org>)^{16,21} as well as our serum IL-18BP data, suggest that IL-18BP may be associated with progression of prostate cancer. Interestingly, IL-18BP in urine after prostate examination (DRE) was elevated in cases with prostate cancer compared to cases without cancer, suggesting a potential role in cancer initiation. IL-18BP was originally identified in urine,⁷ and it is known that initial voided urine obtained after DRE is enriched in prostatic proteins.²⁷ Given that our assays were performed after prostatic manipulation (DRE), that prostatic fluids from extensive prostate cancer have elevated IL-18BP, that only initial urine was collected as it coursed through the prostate after prostatic examination ("Voided bladder 3" samples, per Meares-Stamey),²⁷ and that we normalized to TP in the urine samples (which mostly comes from prostatic sources after a DRE), we surmise that the IL-18BP found in urine after DRE was at least partly if not mostly of prostatic origin. The use of urinary and serum IL-18BP levels for prostate cancer detection, progression, and prognostication would require further, larger scale study.

In summary, our findings of elevated and functional IL-18BP secretion from prostate cancer cells and cell lines suggest a possible attempt by prostate cancer to escape immune surveillance. IL-18BP should be interesting to study further as a prostate cancer marker and therapeutic target.

Acknowledgements

We thank Dr. Robert H. Getzenberg and Dr. Karen Sandell for helpful discussions. We also thank Dr. Alan W. Partin and Leslie A. Mangold for supplying serum samples and Dr. Alan K. Meeker for technical advice in immunohistochemistry.

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